Université de Montréal

Études chimiques et immunologiques des capsules polysaccharidiques de *Streptococcus suis*

par GUILLAUME GOYETTE-DESJARDINS

Département de pathologie et microbiologie
Faculté de médecine vétérinaire

Thèse présentée à la Faculté de médecine vétérinaire
en vue de l'obtention du grade de *Philosophiae Doctor* (Ph.D.)
en sciences vétérinaires option microbiologie

Décembre 2018

Université de Montréal
Faculté de médecine vétérinaire

Cette thèse intitulée

Études chimiques et immunologiques des capsules polysaccharidiques de *Streptococcus suis*

Présentée par
GUILLAUME GOYETTE-DESJARDINS

a été évaluée par un jury composé des personnes suivantes :

Francis Beaudry, président-rapporteur

Mariela Segura, directrice de recherche

Marie-Rose Van Calsteren, codirectrice de recherche

René Roy, codirecteur de recherche

John M. Fairbrother, membre du jury

Martin Lessard, examinateur externe

Marcelo Gottschalk, représentant de la Doyenne de la FÉSP

Résumé

Streptococcus suis est l'un des plus importants pathogènes bactériens du porc, causant des pertes économiques substantielles à l'industrie porcine. De plus, c'est un agent zoonotique représentant de sérieux risques pour la santé humaine. Cette bactérie cause diverses pathologies, dont la méningite, la mort subite et le choc septique sont les plus fréquentes. De plus, il demeure qu'à ce jour, aucun vaccin efficace n'est disponible pour prévenir les infections à *S. suis*.

Étant encapsulé, *S. suis* est classifié en 35 sérotypes définis par l'antigénicité de sa capsule polysaccharidique (CPS). Malgré cela, seule la structure de la CPS du sérotype 2 est connue à présent. Immunologiquement parlant, les CPS purifiées sont généralement des antigènes T-indépendant de par leur nature, faisant d'elles de très pauvres immunogènes. Ceci a déjà été démontré pour la CPS du sérotype 2. Paradoxalement, les anticorps anti-CPS sont fortement opsonisants et protecteurs, ce qui les rend désirables.

Ainsi, les objectifs de cette thèse sont, d'abord, de poursuivre la caractérisation chimique des structures des CPS de différents sérotypes de *S. suis*, puis d'étudier les propriétés immunostimulatrices et immunogènes de ces nouvelles CPS. De plus, les travaux de cette thèse ont tenté d'améliorer l'immunogénicité de la CPS du sérotype 2 pour pouvoir l'utiliser comme antigène vaccinal.

Dans un premier temps, un recensement de la littérature a permis de dresser un portrait à jour et mondial des infections chez le porc et chez l'homme, et d'identifier les sérotypes les plus importants. À l'aide de ces données épidémiologiques, les CPS des sérotypes choisis ont été purifiées, ce qui a permis de déterminer la structure pour huit CPS additionnelles (1, 1/2, 3, 7, 8, 9, 14 et 18).

D'autre part, ces nouvelles connaissances sur la structure nous ont permis d'évaluer l'antigénicité et l'immunogénicité de ces nouvelles CPS. Ainsi, nous avons pu observer pour

la première fois que la CPS du sérotype 3 de *S. suis* est hautement immunogène en produisant une réponse opsonisante d'IgG et d'IgM par un mécanisme T-indépendant.

De plus, nous avons développé pour la première fois un vaccin glycoconjugué constitué de CPS du sérotype 2 couplé au toxoïde tétanique afin d'obtenir une réponse anticorps T-dépendante protectrice. Une preuve de concept chez le porc a permis de démontrer la protection conférée par notre vaccin glycoconjugué contre *S. suis*, et plus largement le potentiel des vaccins glycoconjugués pour combattre les infections bactériennes invasives en médecine vétérinaire. Enfin, l'étude de l'immunogénicité du vaccin glycoconjugué chez la souris a permis d'évaluer le rôle du choix du modèle animal et des adjuvants sur la réponse anti-CPS, en plus de produire trois nouveaux anticorps monoclonaux qui ont permis notamment d'étudier les mécanismes humoraux impliqués dans l'élimination de *S. suis* par opsonophagocytose.

L'ensemble de ces études va permettre de souligner l'importance des anticorps anti-CPS dans la protection face aux infections bactériennes invasives.

Mots-clés : *Streptococcus suis*, sérotype, capsule polysaccharidique, vaccin glycoconjugué, réponse anticorps, opsonophagocytose, structures, immunogénicité, souris, porc

Abstract

Streptococcus suis is one of the most important porcine bacterial pathogens and is responsible for substantial economic losses to the swine industry. Moreover, it is also a zoonotic agent representing serious risks to human health. This bacterium causes a variety of clinical signs, of which meningitis, sudden death, and septic shock are the most frequent. Furthermore, no efficient vaccine is available to protect against infections caused by *S. suis*.

Being encapsulated, *S. suis* is classified into 35 serotypes based on the antigenicity of their capsular polysaccharide (CPS). However, only the structure of the serotype 2 CPS is known to date. Immunologically, purified CPSss generally behave as T-independent antigens, making them very poor immunogens. Indeed, this has been previously demonstrated to be the case for the serotype 2 CPS. Paradoxically, anti-CPS antibodies are strongly opsonizing and protective, making them attractive targets for vaccine development.

Therefore, the objectives of this thesis are, firstly, to further characterize the chemical composition and structure of the *S. suis* CPSs from different serotypes, and secondly, to study the immunostimulatory and immunogenic properties of these additional CPSs. Moreover, this thesis will attempt to increase the serotype 2 CPS immunogenicity for potential use it as a vaccine antigen.

In a first step, a review of the literature provided an updated and global view of *S. suis* infections in swine and humans and identified the most important serotypes. With these epidemiological data in hand, we then purified the CPSs of selected serotypes, which allowed us to determine the structures for eight additional CPSs (1, 1/2, 3, 7, 8, 9, 14, and 18).

The knowledge obtained regarding the structure of theses CPSs allowed us to then evaluate their antigenicity and immunogenicity. We demonstrated for the first time that serotype 3 is highly immunogenic *S. suis* CPS, as it induces an opsonizing response composed of IgG and IgM by a T-independent mechanism.

In addition, we have developped for the first time a glycoconjugate vaccine composed of serotype 2 CPS conjugated to tetanus toxoid in order to obtain a protective T-dependent antibody response. A proof of concept performed with pigs demonstrated protection conferred by our glycoconjugate vaccine against *S. suis*, and more importantly the potential of glycoconjugate vaccines in the fight against invasive bacterial infections in veterinary medicine. Finally, immunogenicity studies performed in mice with this glycoconjugate vaccine allowed us to evaluate the effect of the animal model and adjuvant choice on the anti-CPS response. It also allowed us to produce three new monoclonal antibodies that enabled us to study, among other aspects, the humoral mechanisms involved in *S. suis* clearance by opsonophagocytosis.

Consequently, the work conducted during this thesis will serve to highlight the importance of anti-CPS antibodies in the protection against invasive bacterial diseases.

Keywords : *Streptococcus suis*, serotype, capsular polysaccharide, glycoconjugate vaccine, antibody response, opsonophagocytosis, structures, immunogenicity, mouse, swine

Table des matières

Liste des tableaux

REVUE DE LA LITTÉRATURE

MÉTHODOLOGIE ET RÉSULTATS

Article I. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent – An update on the worldwide distribution based on serotyping and sequence typing

Article II. *Streptococcus suis* serotype 3 and serotype 18 capsular polysaccharides contain di-N-acetyl-bacillosamine

ANNEXES

Liste des figures

REVUE DE LA LITTÉRATURE

MÉTHODOLOGIES ET RÉSULTATS

Article I. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent – An update on the worldwide distribution based on serotyping and sequence typing

Article II. *Streptococcus suis* serotype 3 and serotype 18 capsular polysaccharides contain di-N-acetyl-bacillosamine

Article III. Structure determination of *Streptococcus suis* serotypes 7 and 8 capsular polysaccharides and assignment of functions of the *cps* locus genes involved in their biosynthesis

DISCUSSION

ANNEXES

Article IX. Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide

Article X. Explaining the Serological Characteristics of *Streptococcus suis* Serotypes 1 and 1/2 from their Capsular Polysaccharide Structure and Biosynthesis

Liste des abréviations

6d*xyl*HexNAc	2-acétamido-2,6-didésoxy-β-*xylo*-hexose
AAT-Gal	2-acétamido-4-amino-2,4,6-tridésoxy-D-galactose
Ac	Acétate
ADN	Acide désoxyribonucléique
AID	« Activation-induced cytidine deaminase »
APRIL	« A proliferation inducing ligand »
Ara-ol	Arabitol
ARN	Acide ribonucléique
BAFF	« B cell activating factor »
BBB	« Blood-brain barrier » (barrière hématoencéphalique)
BCR	« B-cell receptor » (récepteur de la cellule B)
BCSFB	« Blood-cerebrospinal fluid barrier » (barrière hémato-liquide céphalorachidien)
BMEC	« Brain microvascular endothelial cell » (cellule endothéliale microvasculaire du cerveau)
BSA	« Bovine serum albumin » (albumine sérique bovine)
CC	Complexe clonal
CCL	« Chemokine (C–C motif) ligand »
CCR	« C–C motif receptor »
CD	« Cluster of differentiation »
CMH-I ou -II	Complexe majeur d'histocompatibilité de classe I ou II
CPA	Cellule présentatrice d'antigène
CPEC	« Choroid plexus epithelial cell » (cellule épithéliale du plexus choroïdien)
CpG ODN	« CpG oligodeoxynucleotide »
CPS	« Capsular polysaccharide » (capsule polysaccharidique)
CR1	« Complement receptor 1 »
CRM_{197}	« Cross-reactive material 197 »
CXCL	« Chemokine (C–X–C motif) ligand »

DC	« Dendritic cell » (cellule dendritique)
DT	« Diphteria toxoid » (toxoïde diphtérique)
EF	« Extracellular factor » (facteur extracellulaire)
ELISA	« Enzyme-linked immunosorbent assay »
ELLA	« Enzyme-linked lectin assay »
Fab	« Antigen-binding fragment »
Fc	« Crystallizable fragment »
FCA	« Freund's complete adjuvant » (adjuvant complet de Freund)
FcγR	« Fcγ receptor » (récepteur Fcγ)
FucNAc	N-acétylfucosamine
FucNAc4N	2-acétamido-4-amino-2,4,6-tridésoxy-D-galactose
Gal	Galactose
GalA	Acide galacturonique
Gal*f*	Galactofuranose
GalNAc	N-acétylgalactosamine
GAS	« Group A *Streptococcus* » (streptocoque du groupe A)
GBS	« Group B *Streptococcus* » (streptocoque du groupe B)
GC	« Germinal center » (centre germinatif)
Glc	Glucose
GlcA	Acide glucuronique
GlcNAc	N-acétylglucosamine
Glc-ol	Glucitol
GM-CSF	« Granulocyte-macrophage colony-stimulating factor »
Gro	Glycérol
IFA	« Incomplete Freund's adjuvant » (adjuvant incomplet de Freund)
IFN	Interféron
IgA	Immunoglobuline de classe A
IgE	Immunoglobuline de classe E
IgG	Immunoglobuline de classe G
IgM	Immunoglobuline de classe M
IL	Interleukine

IRAK	« Interleukin-1 receptor-associated kinase 4 »
ITAM	« Immunoreceptor tyrosine-based activation motif »
ITIM	« Immunoreceptor tyrosine-based inhibition motif »
KLH	« Keyhole limpet hemocyanin »
LPS	Lipopolysaccharide
ManA	Acide mannuronique
ManNAc	N-acétylmannosamine
MAPK	« Mitogen-activated protein kinase » (protéine kinase active par les mitogènes)
MEC	Matricle extracellulaire
mIg	« Membrane immunoglobulin » (immunoglobuline membranaire)
MLST	« Multilocus sequence typing »
MRP	« Muraminidase-released protein »
M_W	« Weight-average molecular mass » (masse molaire moyenne en masse)
MyD88	« Myeloid differentiation primary response 88 »
NCL	« Novel capsular *locus* » (nouveau locus capsulaire)
Neu5Ac	Acide N-acétylneuraminique ou acide sialique
NK	Cellule « natural killer »
NKT	Cellule « natural killer T »
NLRP3	« NOD-like receptor protein 3 »
o/w	« Oil-in-water »
OMP	« Outer membrane protein »
OPA	« Opsonophagocytosis assay » (test d'opsonophagocytose)
P	Phosphate
PAMP	« Pathogen-associated molecular pattern »
PCR	« Polymerase chain reaction »
PCV	« Pneumococcal conjugate vaccine »
PneNAc	N-acétylpneumosamine
PPSV	« Pneumococcal polysaccharide vaccine »
PRR	« Pattern recognition receptor »
PVDF	« Polyvinylidene fluoride »

Py	Pyruvate
QuiNAc	N-acétylquinovosamine
QuiNAc4NAc	di-N-acétylbacillosamine
Rha	Rhamnose
Rib-ol	Ribitol
Sao	« Surface antigen one »
SDS-PAGE	« Sodium dodecyl sulfate–-polyacrylamide gel electrophoresis »
SEM	« Standard error of the mean »
SLY	Suilysine
SNA	« *Sambucus nigra* agglutinin » (lectine de *Sambucus nigra*)
SNC	Système nerveux central
STSLS	« Streptococcal toxic shock-like syndrome »
Tcarb	« T cell that recognize carbohydrates only »
TCR	« T cell receptor » (récepteur des cellules T)
TD	T-dépendant
Th	« T helper cell »
Th1	« Type 1 T helper cell »
Th2	« Type 2 T helper cell »
TI	T-indépendant
TLR	« Toll-like receptor »
TNF	« Tumor necrosis factor »
TRAF	« TNF receptor associated factors »
TRIF	« TIR-domain-containing adapter-inducing interferon-β »
TT	« Tetanus toxoid » (toxoïde tétanique)
Und-P	Undécaprényl-phosphate
w/o	« Water-in-oil »
w/o/w	« Water-in-oil-in-water »

Remerciements

J’aimerais d’abord remercier ma directrice, Dre Mariela Segura, d’avoir en janvier 2010 offert à un jeune biochimiste la chance inouïe de plonger dans l’univers de la recherche. Ensemble, nous avons conçu et mené des projets à la frontière entre la chimie, la biochimie, la glycobiologie et l’immunologie. Compte tenu de mes champs d'intérêt multidisciplinaires et de mes nombreuses questions en recherche, elle a été une directrice remarquable, par son ouverture d’esprit, son ambition, sa patience et son réalisme. Même si je n’ai pas toujours été à la hauteur, elle a droit à toute ma gratitude pour le temps investi, les critiques constructives comme les encouragements, nos discussions scientifiques et personnelles ainsi que les opportunités et conseils offerts. Merci de m’avoir poussé à continuellement me surpasser. En rétrospective, je n’aurais pas pu souhaiter mieux comme formation doctorale et lui en serai toujours reconnaissant. Elle a contribué à façonner le scientifique (et le glycoimmunologiste) que je suis devenu.

J’aimerais aussi remercier ma codirectrice, Dre Marie-Rose Van Calsteren, pour sa présence, sa disponibilité et son implication remarquables tout au long de mes différents projets, particulièrement tout le temps investi, l’accueil chaleureux au sein de son laboratoire, ses conseils et toutes les belles discussions que nous avons eues. Elle aussi a contribué à façonner le scientifique (et le glycobiochimiste) que je suis. Je compte bien avoir le plaisir de la croiser dans une future conférence glyco!

Je sais gré également à mon codirecteur, Dr René Roy, de sa disponibilité et de son implication dans mes différents projets. Je garde un excellent souvenir de l’accueil dans son laboratoire, des discussions que nous avons eues et des précieux conseils qu’il m’a prodigués. Merci d’avoir contribué à façonner le scientifique (et le glycochimiste) que je suis devenu.

Un merci bien particulier au Dr Marcelo Gottschalk : bien qu’il n’ait pas été lié de manière officielle à mes projets, il a joué avec brio le rôle de l’officieux « beau-directeur ». Ce fut un réel privilège de bénéficier de ses conseils et de ses connaissances tout au long de mon Ph.D.

Je n'oublie pas non plus le Dr Hassan Sabik, qui a accepté de me superviser en réponse aux changements survenus au Centre de recherche et de développement sur les aliments d'Agriculture et Agroalimentaire Canada.

Merci aux collaborateurs qui ont contribué à cette thèse et qui m'ont également permis de participer à divers projets : Dre Martine Denicourt, Dr Mario Feldman, Dr Nahuel Fittipaldi, Dr Axel Neubauer, Dr Masatoshi Okura, Dr Daisuke Takamatsu et Dr Evgeny Vinogradov.

Merci aux membres de mon comité-conseil et de mon examen de synthèse pour leurs excellents conseils et le temps qu'ils m'ont consacré : Dre Nathalie Charland, Dr Daniel Dubreuil, Dr Marcelo Gottschalk et Dr Martin Lessard, sans oublier les Dr Francis Beaudry, Dr John Fairbrother et Dr Martin Lessard qui ont accepté de siéger au comité d'évaluation de ma thèse.

Enfin, je remercie les personnes suivantes pour leurs conseils, leur aide précieuse, les belles discussions ainsi que leur amitié tout au long de mes études : Katerine Aubé, technicienne au laboratoire diagnostic de sérologie bactérienne; Dr André Bégin, chercheur au Centre de recherche et de développement sur les aliments; Denis Bélanger, assistant de recherche du Dr Hassan Sabik; Frédéric Berthiaume, coordonnateur de laboratoire à la FMV; Claudia Duquette, technicienne au GREMIP; Fleur Gagnon, assistante de recherche de Dre Marie-Rose Van Calsteren; Nancy Guertin, assistante de recherche de Dre Marie-Rose Van Calsteren; Sonia Lacouture, assistante de recherche du Dr Marcelo Gottschalk; Normand Lacasse, technicien au FANI; Vanessa Lapointe-Sasseville, technicienne au FANI; Geneviève Messier, technicienne au laboratoire diagnostic de sérologie bactérienne; Chichi Shiao, assistant de recherche du Dr René Roy; et Line Vachon, technicienne au laboratoire diagnostic de sérologie bactérienne. Un petit mot pour également remercier Agustina Lavagna pour ses talents de graphiste.

Je salue tous mes collègues de laboratoire, anciens et présents, avec qui j'ai eu la chance de collaborer au fil des années. Un merci plus particulier à Cynthia, Paul, David, Audrey D., Corinne, Léa, Annabelle, Agustina L., Dominic (ceci n'est pas un clin d'œil!), Anaïs et Annie pour votre aide, votre amitié, les moments de fous rires, votre soutien, vos encouragements, vos bons conseils et aussi vos questions de chimie. Merci également à Adamy, Agustina S., Claire-Océanne, Claude, Damian, Dang, Darielys, Gaël, Jessica, Lorelei, Madeleine, Mar, Marêva, Mariela, Marie-Pier, Sarah C., Sarah F., Shujie et Yingchao. De plus, merci aux stagiaires que j'ai eu la chance de superviser : Adrien, Camille, Élise, Audrey G., Évelyne et Mariane. À vous tous, bonne chance dans vos études et, de surcroît, tout le succès que vous méritez dans vos futures carrières! Au plaisir de se recroiser et, qui sait, de collaborer…

J'aimerais aussi mentionner tous les professeurs et membres du GREMIP, du CRIPA et de la Faculté de médecine vétérinaire. Un merci plus particulier pour l'aide précieuse et chaleureuse de Nancy Bisaillon, Christine Blondin, Hélène Boucher-Rhéaume, Manon Coutellier, Cécile Crost, Isabelle Flibotte, Geneviève Lauzon, Diane Rodier et Micheline St-Germain.

Tout au long de mon doctorat, j'ai eu le privilège de recevoir des bourses des organismes suivants pour le soutien du projet ainsi que la chance de participer à plusieurs congrès scientifiques : Conseil de recherches en sciences naturelles et en génie du Canada (CRSNG), Fonds de recherche du Québec – Nature et technologies (FRQNT), Canadian Glycomics Network (GlycoNet), Centre de recherche en infectiologie porcine et avicole (CRIPA), Direction des affaires internationales (DAI) de l'Université de Montréal, Faculté des études supérieures et postdoctorales (FESP), Faculté de médecine vétérinaire (FMV), Cité de la biotechnologie agroalimentaire, vétérinaire et agroenvironnementale de Saint-Hyacinthe et Fondation Lucie Besner.

Un si long parcours aurait évidemment été impossible sans le soutien et l'accompagnement de ma famille et de tous les amis qui m'ont soutenu durant toutes ces années et qui n'ont jamais cessé de s'intéresser à ma recherche. Je remercie plus

particulièrement mes parents pour les valeurs transmises, notamment le dépassement de soi, le goût du travail bien fait, le dévouement et le courage de poursuivre ses ambitions : sans tenir compte de la génétique, vous avez droit à une part de crédit pour l'homme d'aujourd'hui.

Tout ce cheminement aurait été impossible sans la présence à mes côtés de Jean-Philippe qui a été, depuis nos premières semaines de baccalauréat à Sherbrooke, mon rayon de soleil quotidien. Il a toujours su être là pour moi (même au-delà de l'Atlantique), à m'encourager, me supporter et me réconforter dans les moments autant chaotiques que paisibles. Couvert de pansements de la tête aux mains, il a pratiquement laissé sa peau au laboratoire, un exemple de passion et de dévouement que j'admire et cherche à suivre. Ce parcours rocambolesque, nous l'avons fait ensemble, d'égal à égal, jusqu'au royaume de l'autobahn. Merci pour le passé, merci pour le présent, et merci d'avance pour le futur. Du fond du coeur, merci.

Enfin, je ne saurais passer sous silence la contribution de tous les porcs et les souris qui m'ont permis d'effectuer ces études. L'expérimentation animale est un privilège, et j'ai une dette à leur égard.

I. INTRODUCTION

Streptococcus suis est un important pathogène porcin causant de sévères problèmes économiques dans l'industrie du porc. Cet agent zoonotique représente également un sérieux risque pour la santé publique. Étant une bactérie extracellulaire encapsulée, *S. suis* est classifié en 35 sérotypes selon l'antigénicité de sa capsule polysaccharidique (CPS). Cependant, à l'exception de la structure de la CPS du sérotype 2 qui a précédemment été décrite, les structures des CPS pour tous les autres sérotypes demeurent inconnues. En l'absence de ces connaissances, les différentes réactions croisées qui surviennent lors de la sérotypie de *S. suis* demeurent inexplicables.

De plus, la CPS de *S. suis* est reconnue comme étant le seul facteur de virulence critique. En masquant les antigènes et les protéines de surface, et en étant un important facteur anti-phagocytaire, la CPS permet à *S. suis* d'échapper aux défenses du système immunitaire de l'hôte infecté, autant à la réponse innée qu'à la réponse adaptative où la capsule démontre des fonctions immunomodulatrices lors de la réponse inflammatoire et en prévenant le développement d'une réponse adaptative protectrice, notamment par le fait que les polysaccharides sont reconnus comme étant très pauvrement immunogènes de par leur nature d'antigène T-indépendant (TI), tel que déjà démontré pour la CPS du sérotype 2 de *S. suis*.

Compte tenu de la faible immunogénicité de *S. suis*, mais également de sa très grande diversité génotypique et phénotypique parmi les souches, il n'est pas surprenant de constater qu'il n'existe encore aucun vaccin efficace pour prévenir les infections à *S. suis*, principalement chez le porc. Également, aucun vaccin basé sur la (les) CPS de *S. suis* n'a été décrit à ce jour.

Malgré les connaissances très limitées sur les structures des CPS de *S. suis* et leurs propriétés antigéniques et immunogènes, un vaccin basé sur la CPS de *S. suis* peut offrir de nombreux avantages : d'abord, la CPS étant la couche la plus externe de l'enveloppe bactérienne, les anticorps la ciblant sont connus pour être hautement opsonisants et protecteurs. De plus, les anticorps dirigés contre la CPS offrent une protection pan-sérotype contre toutes les souches encapsulées de ce sérotype. Ainsi, le développement d'un vaccin basé sur la (les) CPS(s) de *S. suis* comporte un potentiel énorme pour l'obtention de vaccins

protecteurs et efficaces, mais présente également des défis considérables. Une avenue intéressante à explorer sont les vaccins glycoconjugués, où un antigène polysaccharidique (TI) est couplé de façon covalente à une protéine porteuse qui permettrait l'obtention d'une puissante réponse opsonisante anti-CPS T-dépendante (TD) avec commutation isotypique, maturation d'affinité et développement d'une mémoire immunologique. La vaccination par l'aide d'un vaccin glycoconjugué permettrait une forte production d'anticorps protecteurs qui permettront l'élimination de *S. suis* par opsonophagocytose. En opsonisant la bactérie dès son arrivée dans la circulation, cela préviendrait l'initiation de l'infection par *S. suis* et l'apparition des signes cliniques.

C'est ainsi que l'**hypothèse générale** de cette thèse est que les anticorps anti-CPS protègent l'hôte en permettant l'élimination de *S. suis*, un pathogène extracellulaire encapsulé, par opsonophagocytose. Plus spécifiquement :

i. Ces anticorps sont également protecteurs contre toute souche d'un même sérotype, surmontant ainsi la grande variation phénotypique parmi celles-ci.
ii. Les CPS de *S. suis* sont des candidats idéaux pour le développement de vaccins efficaces pour la prévention de cette maladie.
iii. Ces CPS n'ont pas toutes les mêmes propriétés immunogènes, compte tenu de leurs différences compositionnelles et structurales.
iv. La création et l'emploi d'un vaccin glycoconjugué préparé à partir d'une CPS renversera son caractère non immunogène par la génération d'une réponse TD.

Les **objectifs généraux** de cette thèse sont, d'abord, de poursuivre la caractérisation des structures des CPS de *S. suis*, puis d'étudier les interactions entre celles-ci et le système immunitaire de l'hôte dans le cadre du développement d'une réponse humorale protectrice.

Ainsi, les **objectifs spécifiques** se déclinent selon les deux grands axes de recherche suivants :

AXE I – Diversité des capsules polysaccharidiques de *S. suis*

1. Recenser les sérotypes de *S. suis* impliqués lors d'infections chez le porc et chez l'homme dans la littérature.

2. Déterminer les structures des CPS pour les sérotypes importants de *S. suis* par une approche de chimie structurale (analyses chimiques et spectroscopiques).

   - Pour ces deux premiers objectifs, grâce à l'élucidation des structures des CPS pour les sérotypes les plus importants de *S. suis*, nous seront alors en mesure d'étudier leurs propriétés antigéniques et immunogènes tel que proposé dans le second axe.

AXE II – Réponse immunitaire humorale face aux capsules polysaccharidiques de *S. suis*

3. Caractériser l'antigénicité des CPS de *S. suis* dont la structure est connue et d'évaluer leur immunogénicité en évaluant la réponse humorale anti-CPS. Pour ce faire, les sous-objectifs suivants ont été établis :
   3.1. Caractériser l'antigénicité de ces CPS purifiées en évaluant les éléments structuraux reconnus par les sérums de sérotypie à l'aide de techniques sérologiques.
   3.2. Évaluer le pouvoir activateur de ces CPS purifiées face à des DCs par des stimulations *in vitro* en caractérisant la réponse inflammatoire et le rôle des TLR.
   3.3. Évaluer l'immunogénicité de ces CPS en caractérisant la nature et la fonctionnalité de la réponse humorale anti-CPS suite à des immunisations *in vivo* à l'aide de CPS purifiées, de bactéries entières tuées et de bactéries vivantes (infections à dose sous-létale).

   - Pour ce troisième objectif, grâce aux CPS purifiées obtenues lors du premier axe, nous serons en mesure de mieux comprendre les réactions croisées obtenues lors du sérotypage de *S. suis*, ainsi que d'évaluer le potentiel des CPS purifiées sur la stimulation de CPA et l'induction de réponses humorales anti-CPS.

4. Créer un prototype de vaccin glycoconjugué dirigé contre le sérotype 2 de *S. suis* et à en faire la preuve de concept chez le porc (volet appliqué) en caractérisant la nature et la protection de la réponse humorale anti-CPS suite à une immunisation *in vivo*.

   - Pour ce quatrième objectif, la création d'un vaccin glycoconjugué et son utilisation permettra de remédier au caractère non-immunogène de la CPS du sérotype 2 par l'induction d'une réponse anticorps protectrice chez le porc, démontrant le potentiel de ce type de vaccin pour la médecine vétérinaire.

5. Évaluer l'immunogénicité d'un vaccin glycoconjugué contre le sérotype 2 de *S. suis* et la fonctionnalité des anticorps produits, ainsi que l'influence du modèle expérimental sur ceux-ci. Pour ce faire, les sous-objectifs suivants ont été établis :
   5.1. Caractériser la nature et la fonctionnalité de la réponse humorale induite par un vaccin glycoconjugué dirigé contre la CPS du sérotype 2 de *S. suis* suite à des immunisations *in vivo*.
   5.2. Étudier l'influence du choix de l'adjuvant et du modèle animal sur l'immunogénicité du glycoconjugué en caractérisant la nature et la fonctionnalité de la réponse humorale anti-CPS.
   5.3. Obtenir des anticorps monoclonaux anti-CPS obtenus grâce au glycoconjugué, puis étudier leur spécificité et leur potentiel protecteur par l'emploi de techniques sérologiques et d'essais fonctionnels *in vitro* et *in vivo*.

   - Pour ce cinquième objectif, l'étude plus détaillée des facteurs influençant la réponse immunitaire face au glycoconjugué et des propriétés de celle-ci permettra de mieux comprendre la complexité de la CPS du sérotype 2 de *S. suis* couplée à une protéine porteuse comme antigène vaccinal.

II. REVUE DE LITTÉRATURE

1. *Streptococcus suis*

1.1. Caractéristiques générales

S. suis est une bactérie encapsulée à Gram positif en forme de coque, fréquemment retrouvée sous forme isolée ou en paire, et plus rarement en courtes chaînes, d'un diamètre inférieur à 2 µm (1). De plus, *S. suis* est anaérobie facultative, non motile et forme des colonies mucoïdes gris-blanc α-hémolytiques sur gélose supplémentée de sang de mouton (2).

1.2. *Streptococcus suis* chez le porc

S. suis est une bactérie commensale des voies respiratoires supérieures des porcs, plus particulièrement retrouvée au niveau des cavités nasales et des amygdales, mais peut également être retrouvée dans les tractus génitaux et gastro-intestinaux (3, 4). En effet, on considère que 100% des porcs sont naturellement colonisés par au moins un sérotype de *S. suis*, bien que la présence de plusieurs sérotypes est fréquente (1, 5). Toutefois, bien que le porc soit un porteur asymptomatique de *S. suis*, cette bactérie est la cause d'infections graves entraînant des pertes économiques importantes à l'industrie porcine, et ce mondialement (1). Cependant, bien que différentes souches appartenant à un seul ou plusieurs sérotypes peuvent être retrouvées parmi les animaux porteurs, normalement seule une souche est responsable de l'apparition de la majorité des cas cliniques au sein d'un troupeau donné (5).

1.2.1. Transmission et facteurs de risques

S. suis peut se transmettre de manière verticale et/ou horizontale. La transmission verticale se fait durant la mise bas, lorsque les porcelets sont en contact direct avec les bactéries présentes dans les sécrétions vaginales (3). De plus, la bactérie se transmet de manière horizontale par des contacts entre les porcelets ou avec la truie (3). À ce jour, différents facteurs de risque ont été identifiés comme pouvant influencer le développement des infections à *S. suis* à la ferme (3). En effet, certaines pratiques d'élevage pouvant contribuer au stress des animaux, dont des fluctuations excessives de température, l'humidité relative élevée,

la mauvaise ventilation, l'entassement, l'écart d'âge entre les porcelets à l'intérieur d'une même pièce, ainsi que le stress lié au transport et à la vaccination constituent des facteurs augmentant la susceptibilité des animaux à l'infection (3). De plus, le statut immunitaire du troupeau et la présence d'autres pathogènes, tels que le virus du syndrome reproducteur et respiratoire porcin et les mycoplasmes, peuvent augmenter les risques d'une infection à *S. suis* (6, 7).

1.2.2. Signes cliniques et pathologies associées

S. suis peut causer une variété d'infections chez le porc, dont la mort subite, la méningite, l'arthrite et l'endocardite sont les plus fréquentes (8, 9). La plupart des manifestations cliniques surviennent entre cinq et dix semaines d'âge, étant donné que les porcelets sont plus susceptibles à développer une infection suite au sevrage en raison des différents stress qu'ils y subissent (séparation de la mère, fin de l'immunité passive maternelle et délai dans l'acquisition d'une immunité active, changements liés à leur environnement et à leur diète, etc.) (10).

Un des premiers signes observés chez les animaux malades à *S. suis* est une hyperthermie accompagnée d'une importante perte de poids pour la majorité des cas et de l'apparition de signes de dépression (3). On peut également retrouver la bactérie dans la circulation sanguine où elle pourra causer le sepsis si l'animal n'est pas traité rapidement (3). Différents signes cliniques neurologiques peuvent être observés chez les animaux atteints de méningites, parmi lesquels il est possible de retrouver l'ataxie, l'opisthotonos, les convulsions et le nystagmus (3). Pour leur part, les animaux atteints d'autres pathologies, telles que des endocardites, présenteront plutôt des signes de dyspnée respiratoire, de cyanose et de dépression (3).

1.3. Infections zoonotiques causées par *Streptococcus suis*

En plus d'être un important pathogène chez le porc, *S. suis* est aussi un agent de zoonose causant principalement méningites et chocs septiques chez l'humain (8). Depuis le premier cas d'infection humaine rapporté au Danemark en 1968, plus de 1 600 cas ont été décrits à ce jour (**ARTICLE I**). En effet, le nombre de cas rapportés a augmenté de manière considérable dans les dernières années, particulièrement dans les pays d'Asie, où deux éclosions mortelles ont eu lieu en Chine en 1998 et en 2005 (1). En Amérique du Nord et en Europe, les infections humaines à *S. suis* sont considérées comme étant une maladie professionnelle, c'est-à-dire que les travailleurs de l'industrie porcine, les éleveurs, les vétérinaires, les employés d'abattoirs ainsi que les bouchers sont les plus à risque de développer la maladie suite au contact de lésions cutanées avec un animal infecté (1, 8). Cependant, en Asie, et plus particulièrement au Vietnam et en Thaïlande, la consommation d'aliments crus ou peu cuits, dérivés de porcs malades ou préparés à partir de viande contaminée à *S. suis*, est une cause importante de la propagation de *S. suis* (8). La rareté des infections chez l'homme suggère toutefois que d'autres facteurs sont impliqués, tels que notamment l'immunosuppression, le diabète, l'alcoolisme, l'asplénie et le cancer (11).

Le nombre croissant d'infections humaines à *S. suis* a considérablement augmenté l'intérêt pour ce pathogène, particulièrement à la suite de l'éclosion de 2005 (8). Non seulement le nombre d'articles scientifiques publiés à son sujet a significativement augmenté depuis le début des années 2000, mais les problèmes que ce pathogène représente pour la médecine humaine et la santé publique ont contribué à améliorer la surveillance et les méthodes diagnostiques en Asie (8). Toutefois, la prévalence en Asie reste beaucoup plus élevée que celle en Europe et en Amérique du Nord. De plus, les taux de mortalité varient de moins de 3% dans la plupart des pays occidentaux à 26% dans certains pays asiatiques (**ARTICLE I**).

1.3.1. Signes cliniques et pathologies associées

Chez l'humain, *S. suis* cause une infection systémique à la suite d'une période d'incubation variant de quelques heures à deux jours (8). Comme chez le porc, la méningite est la manifestation clinique la plus fréquente, mais le développement de choc septique est également très commun (12). De plus, il est à noter que des centaines de cas du syndrome apparenté au choc toxique streptococcique (STSLS) ont été associés à la souche responsable des éclosions en Chine (sérotype 2) (12). En plus d'une fièvre élevée, ce syndrome est caractérisé par frissonnement, mal de tête, vomissement, douleur abdominale, et par des signes cliniques d'hypotension, de tachycardie, de dysfonction hépatique, de coagulation intravasculaire disséminée, de défaillance rénale aiguë et de syndrome de détresse respiratoire aiguë (13). Alors que la mort survient souvent lors d'un choc septique, la complication la plus fréquente suite à une méningite par *S. suis* est la surdité, temporaire ou permanente et qui peut affecter une ou les deux oreilles, à différents degrés d'atteinte (13, 14). De plus, ces séquelles de surdité surviennent lors d'environ un cas sur deux.

1.4. Typage

Deux grandes approches de typage sont couramment utilisées pour caractériser les souches de *S. suis*, soit le typage sérologique, qui permet de différencier les souches selon l'antigénicité de leur CPS, et le typage allélique par « multilocus sequence typing » (MLST) permettant de suivre l'évolution phylogénétique de différentes souches (**ARTICLE I**).

1.4.1. Tests sérologiques et sérotypes décrits

Plusieurs tests peuvent être employés en laboratoire pour observer la reconnaissance d'une souche donnée par un antisérum de référence (15). De par sa simplicité et son aisance à lire les résultats à l'œil (sans instrument requis tel qu'un microscope), la majorité des laboratoires diagnostiques préfèrent le test sérologique de coagglutination (12). Le principe derrière ce test repose sur la protéine A, exprimée à la surface de *Staphylococcus aureus* et capable de lier la portion « Crystallizable fragment » (Fc) d'une immunoglobuline de classe G (IgG), laissant sa portion « antigen-binding fragment » (Fab) libre de lier l'antigène spécifique

(16). Ainsi, pour préparer le réactif de coagglutination, un antisérum de référence de lapin (préparé par hyperimmunisation avec la souche de référence pour le sérotype voulu) sera au préalable mélangé avec une culture tuée de *S. aureus*, ce qui permettra aux IgGs de s'adsorber à la surface de *S. aureus* (grâce à la protéine A). Un réactif de coagglutination doit être préparé pour les antisérums spécifiques pour chaque sérotype. Enfin, pour sérotyper une souche, il suffit de mélanger ensemble une goutte de culture tuée de *S. suis* avec une goutte du réactif de coagglutination, et d'attendre jusqu'à 1–2 minutes. Si la réaction est positive, des aggrégats apparaîtront puisque les bactéries de *S. suis* et de *S. aureus* (avec IgG anti-CPS de *S. suis* adsorbés à sa surface) auront agglutinés ensembles (16).

À ce jour, 35 sérotypes différents de *S. suis* ont été établis en fonction de l'antigénicité de la CPS, soit les sérotypes 1 à 34 et le sérotype 1/2 (15, 17-20). Bien que cette méthode soit relativement fiable, le sérotypage comporte certaines limites, dont la présence de réactions croisées (**Tableau I**) et l'existence de souches non typables ou auto-agglutinantes (12, 21-24). De plus, les avancées en génétique et biologie moléculaire ont permis de démontrer que les sérotypes 20, 22, 26, 32, 33 et 34 appartiennent à des espèces autres que *S. suis* (25-28). Ainsi, les sérotypes 20, 22 et 26 appartiendraient à *Streptococcus parasuis* (29), le sérotype 33 à *Streptococcus ruminantium* (30) et les sérotypes 32 et 34 à *Streptococcus orisratti* (31). Au commencement de ce projet de doctorat, seule la structure de la CPS du sérotype 2 avait été déterminée (voir plus loin). De ce fait, aucune information n'était encore disponible pour expliquer les différentes réactions croisées observées en sérotypie.

Tableau I. Sérotypes de *S. suis* produisant des réactions croisées lors de la sérotypie (15, 17).

Sérotypes	
1	14
1/2	1
1/2	2
2	22
6	16

1.4.2. Sérotypage moléculaire

Afin de contourner les inconvénients des tests sérologiques, dont l'utilisation d'animaux pour préparer les antisérums, des tests moléculaires basés sur l'amplification de gènes spécifiques au sérotype ont été développés (28). Pour chacun des sérotypes, une séquence unique située soit dans le gène codant pour la polymérase (Wzy) impliquée dans la biosynthèse de la CPS ou dans une glycosyltransférase est ciblée, à l'exception des sérotypes 1 et 14 et les sérotypes 1/2 et 2, qui ne peuvent pas être différenciés à l'aide de cette méthode (32, 33). Toutefois, certaines souches non sérotypables demeurent non typables par ces techniques d'identification moléculaires. Ce phénomène est normalement indicateur de nouveaux sérotypes jusqu'alors non décrits : depuis 2015, il a été proposé le nouveau sérotype Chz (34) en plus de 17 « Novel CPS *loci* » définis suite au séquençage des locus capsulaires de souches non sérotypables (24, 35, 36) (voir plus loin).

1.4.3. Typage allélique par « multilocus sequence typing »

Le typage moléculaire allélique ou « multitlocus sequence typing » (MLST) est une technique permettant la caractérisation de souches au sein d'une espèce bactérienne en se basant sur la séquence interne de sept gènes hautement conservés (gènes de ménage) (37).

Pour *S. suis*, le MLST se base sur des variations dans les gènes *aroA* (gène codant pour la 5-énolpyruvylshikimate-3-phosphate synthétase), *cpn60* (gène codant pour une chaperonne de 60 kDa), *dpr* (gène codant pour une protéine putative de résistance au peroxyde), *gki* (gène codant pour une glucose kinase), *mutS* (gène codant pour une enzyme de réparation des erreurs dans l'acide désoxyribonucléique [ADN]), *recA* (gène codant pour un facteur de recombinaison homologue) et *thrA* (gène codant pour une aspartokinase/homosérine déshydrogénase). Chaque séquence interne des gènes, appelée allèle, est associée à un numéro afin de créer un profil composé des sept allèles des gènes cibles. Ce profil est ensuite associé à une séquence type allélique (ST) précise (38).

Cette technique ne considère pas l'ensemble des polymorphismes nucléotidiques de la séquence, mais cible plutôt quelques variations précises au sein de la séquence (37). Elle permet d'avoir une vue d'ensemble de l'évolution de la bactérie et de mieux comprendre l'évolution phylogénétique de *S. suis* (38). Cette méthode a suscité beaucoup d'intérêt au cours des dernières années et est fréquemment utilisée depuis la disponibilité des technologies de séquençage, ainsi que de l'accessibilité de la banque d'allèles et de profils en ligne (12). Néanmoins, le MLST comporte certaines limitations. Entre autres, l'utilisation de gènes de ménage peut masquer des différences génotypiques, et par conséquent phénotypiques, importantes entre les souches. L'acquisition récente d'ADN exogène de phages, d'îlots génomiques, d'éléments intégratifs et conjugatifs ou des phénomènes de recombinaison homologue pourraient ainsi passer inaperçus (37).

À ce jour, 1 096 différentes STs de *S. suis* ont été rapportés. Cependant, plusieurs STs sont étroitement reliés, suggérant un ancêtre commun (12). Il est suggéré qu'une association entre la virulence d'une souche et la ST pourrait exister, du moins pour le sérotype 2 (39). En effet, la ST1a (prédominante en Europe, en Asie et en Amérique du Sud) et la ST7 (présente en Chine et responsable des éclosions humaines de 1998 et 2005) est fortement associé aux cas d'infections sévères caractérisées par le sepsis, la méningite ou l'arthrite (12). Au contraire, la ST25 et la ST28, fréquemment retrouvées en Amérique du Nord, sont associées avec une virulence intermédiaire et faible, respectivement (39). De plus, certaines STs sont associées à la présence de gènes codant pour certains marqueurs de virulence comme la

suilysine (*sly*), la « muraminidase-released protein » (MRP) (*mrp*) et le facteur extracellulaire (EF) (*epf*) (39). Les souches appartenant au ST1 et au ST7 possèdent les marqueurs de virulence *sly, mrp* et *epf* alors que les ST25 et ST28 sont négatives pour *sly* et *epf*, avec la présence ou l'absence de *mrp* permettant de distinguer entre ces deux STs (39). Curieusement, les souches d'un même sérotype peuvent être regroupées en plusieurs STs, de même qu'une ST particulière peut être composée de souches de sérotypes différents (12).

1.5. Épidémiologie

Bien qu'il existe une multitude d'études rapportant l'épidémiologie des souches de *S. suis* retrouvées soit au niveau des voies respiratoires de porcs en santé (porteurs sains), au sein des abattoirs ou impliquées dans les infections cliniques chez le porc pour une grande partie des pays / régions du monde, aucun portrait global à jour n'était disponible au commencement de ce projet de doctorat. De plus, pour ce qui est des infections à *S. suis* chez l'homme, malgré l'existence d'excellentes revues de littératures compilant les cas humains et rapportant les caractéristiques cliniques de ces infections (1, 40, 41), il n'existait aucun recensement des sérotypes impliqués au commencement de ce projet de doctorat.

1.6. Pathogenèse de l'infection et réponse inflammatoire

Afin de pouvoir causer son infection, *S. suis* doit traverser plusieurs barrières physiques et immunitaires (42). En particulier, la bactérie doit d'abord coloniser, puis envahir l'épithélium, afin d'atteindre la circulation sanguine. Une fois dans le sang, la bactérie doit résister à l'activité bactéricide des leucocytes, afin de pouvoir se multiplier, persister et se disséminer. Ceci lui permet alors de causer une infection systémique caractérisée par le sepsis et, suite à l'induction d'une production exacerbée de médiateurs pro-inflammatoires, le choc septique. Si l'hôte survit à l'infection systémique, *S. suis* peut franchir l'une des deux barrières séparant le système nerveux central (SNC) du sang, soit la barrière hématoencéphalique (BBB) ou la barrière hémato-liquide céphalorachidien (BCSFB). Une fois dans le SNC, *S. suis* cause une infection locale caractérisée par une réponse inflammatoire exacerbée, menant au

développement de la méningite accompagnée ou non d'une encéphalite (méningo-encéphalite) (42).

1.6.1. Voies d'entrée chez l'hôte et colonisation des amygdales chez le porc

La voie d'entrée la plus commune chez le porc est via les voies respiratoires supérieures, où la bactérie colonise les amygdales palatines et pharyngées (43, 44). Les amygdales sont décrites comme étant un des sites primaires d'invasion bactérienne, suivie des nodules lymphatiques pharyngés (43). En effet, des antigènes de *S. suis* y ont été identifiés et des bactéries entières sont fréquemment retrouvées dans les cryptes luménales et dans l'épithélium des zones subépithéliales immédiates (43).

Toutefois, le tractus gastro-intestinal ne peut pas être exclu comme site d'infection secondaire, puisque les intestins des porcelets sont rapidement colonisés lors du sevrage (45). Tel que mentionné précédemment, les voies d'entrée principales chez l'humain sont soit par l'entremise de lésions cutanées suite au contact avec des porcs malades ou avec leurs dérivés, ou au niveau intestinal suite à l'ingestion de produits contaminés crus ou peu cuits (45). À ce jour, l'entrée par les voies respiratoires n'a jamais été démontrée chez l'humain (45).

1.6.2. Adhésion et invasion de l'épithélium

Les mécanismes par lesquels *S. suis* parvient à franchir l'épithélium demeurent encore peu connus à ce jour (45). En effet, *S. suis* adhère et envahit faiblement les cellules épithéliales *in vitro* (42). De plus, la production de biofilm par *S. suis*, afin de survivre et coloniser les amygdales, demeure controversée (45). Toutefois, l'adhésion de *S. suis* aux cellules épithéliales dépend de différentes adhésines, qui sont partiellement masquées par la présence de la CPS (45). En effet, plus de vingt facteurs ont été décrits comme participant à l'adhésion et à l'invasion des cellules épithéliales par *S. suis* (42, 46-61). Ainsi, il a été proposé que *S. suis* peut réguler à la baisse l'expression de sa CPS durant les premières étapes de l'infection, afin de favoriser les interactions avec ces cellules (45). En effet, seules les souches non encapsulées ont la capacité d'envahir les cellules épithéliales (42). De plus, la suilysine (SLY)

(une toxine produite par certaines souches de *S. suis*) pourrait aussi participer dans la perturbation de la surface épithéliale avec son activité cytotoxique (62). Une fois la barrière épithéliale franchie, *S. suis* peut ensuite interagir avec la matrice extracellulaire (MEC) (42). Plus de quarante protéines ont été décrites comme ayant un rôle dans la liaison de *S. suis* à la MEC, que ce soit à la fibronectine, au fibrinogène, à la laminine ou encore au collagène (63). L'adhésion de *S. suis* aux protéines de la MEC pourrait faciliter les interactions avec les cellules épithéliales en plus de l'invasion du tissu sous-jacent (45).

1.6.3. Survie dans le sang et dissémination systémique

Une fois l'épithélium franchi, *S. suis* se retrouve alors en circulation sanguine. Dans le sang, *S. suis* circule par l'intermédiaire de facteurs encore inconnus, soit librement ou lié à la surface des monocytes, selon l'hypothèse du cheval de Troie modifié (42, 62, 64). *S. suis* possède plusieurs facteurs lui permettant de résister à la phagocytose et à l'effet bactéricide des neutrophiles et des monocytes sanguins (42). En effet, la CPS est un facteur critique permettant à *S. suis* de persister dans le sang (42, 65, 66). Contrairement aux étapes d'adhésion et d'invasion des surfaces épithéliales, la présence de la CPS est nécessaire pour la survie bactérienne dans le sang, comme il a été démontré par l'utilisation de souches non encapsulées. En effet, celles-ci, contrairement à leur souche-mère, sont rapidement éliminées de la circulation sanguine (42, 67, 68), notamment par phagocytose. De plus, il a été suggéré que *S. suis* peut réguler à la hausse l'expression de sa CPS durant cette étape (42). Plusieurs autres facteurs sont également impliqués dans la tolérance au stress oxydatif (42). De plus, la SLY cause le relâchement d'hémoglobine des érythrocytes, contribuant à l'augmentation des niveaux de médiateurs pro-inflammatoires, en agissant en synergie avec des composants de la paroi cellulaire de *S. suis* (69). Ensemble, ces mécanismes permettent à *S. suis* de se multiplier, de persister et de se disséminer, causant ainsi une bactériémie pouvant mener au sepsis (42).

1.6.4. Activation de la réponse inflammatoire systémique et choc septique

Bien que l'activation de l'immunité innée soit protectrice pour l'hôte lors d'une infection bactérienne, un choc septique peut se développer suite à une régulation déséquilibrée de cette réponse (70). Le relâchement excessif de médiateurs pro-inflammatoires peut être dommageable pour l'hôte, car cela perturbe l'homéostasie vasculaire, ce qui peut conduire à une dysfonction multiorganes (70). En effet, la persistance de *S. suis* dans le sang et sa dissémination dans différents organes internes (particulièrement le foie, la rate, le cœur et les reins), cause une activation rapide et déséquilibrée des cellules de la réponse immunitaire innée (les neutrophiles, les monocytes et les cellules « natural killer » [NK] dans le sang et les macrophages et les cellules dendritiques [DCs] dans les organes) et l'induction massive de médiateurs pro-inflammatoires menant directement au développement du choc septique (42, 71-74). De plus, certaines souches virulentes, dont celles responsables des éclosions en Chine caractérisées par des cas de STSLS, manipulent à leur avantage le système immunitaire de l'hôte (75). Ainsi, *S. suis* induit non seulement une production élevée de médiateurs pro-inflammatoires par les cellules porcines, murines et humaines *in vitro* (42), mais également des niveaux systémiques élevés de médiateurs pro-inflammatoires *in vivo* durant le choc septique chez la souris, le porc et l'humain, dont le facteur de nécrose tumoral (TNF), l'interleukine (IL) 6, l'IL-12p70, l'interféron (IFN) γ, le « C–C motif ligand » (CCL) 2, le CCL3, le CCL5 et le « C–X–C motif ligand » (CXCL) 1 (76-79). De plus, *S. suis* induit l'expression de molécules d'adhésion impliquées dans l'inflammation, provoquant ainsi le recrutement massif de leucocytes et l'amplification de la réponse inflammatoire (42). L'activation cellulaire par *S. suis* implique des récepteurs de reconnaissance et des motifs moléculaires, notamment le CD14 et le TLR2, mais d'autres TLRs pourraient aussi intervenir (42). Des études à l'aide de mutants non encapsulés, ont démontré le rôle des composants de la paroi bactérienne dans l'induction de la réponse inflammatoire : bien que ces composants impliqués soient peu caractérisés, les lipoprotéines à la surface de la bactérie sont fort probablement impliquées (42, 80, 81).

1.6.5. Voies d'entrée dans le système nerveux central

Si l'hôte survit à l'infection systémique causée par *S. suis*, il est susceptible de développer une infection du SNC, lorsque la bactérie atteint celui-ci. Pour ce faire, la bactérie doit traverser la BBB ou la BCSFB. Toutefois, les mécanismes utilisés par *S. suis* pour envahir le SNC restent nébuleux à ce jour (42).

1.6.5.1. La barrière hématoencéphalique

La BBB est une barrière structurelle et fonctionnelle formée par les cellules endothéliales microvasculaires du cerveau (BMEC). Les BMECs possèdent plusieurs caractéristiques, dont la présence de jonctions serrées, et ont une résistance électrique élevée et un faible taux de pinocytose (82, 83). La BBB fonctionne comme barrière hautement résistante aux macromolécules circulantes, protégeant ainsi le cerveau des microorganismes et des toxines pouvant circuler dans le sang. Une perturbation de la BBB est une caractéristique de la pathophysiologie de la méningite bactérienne (82, 84). Ainsi, différents facteurs impliqués dans la virulence de *S. suis* ont été caractérisés comme participant dans l'adhésion et l'invasion des BMECs porcines (42). Contrairement à son interaction avec les cellules épithéliales, *S. suis* peut envahir les BMECs porcines et y survivre de manière intracellulaire jusqu'à 7 heures, ce qui pourrait être un élément crucial pour traverser la BBB et favoriser le développement de la méningite (85). Une fois de plus, la SLY participe à l'invasion des BMECs par son activité hémolytique (42). Plus récemment, il a été démontré que l'énolase de *S. suis* possède la capacité d'augmenter la perméabilité de la BBB (86). De plus, certaines composantes du sérum peuvent également participer dans les interactions entre *S. suis* et les BMECs porcines : parmi celles-ci, seule la fibronectine a été démontrée comme jouant un important rôle (42).

1.6.5.2. La barrière hémato-liquide céphalorachidien

Le plexus choroïdien est composé d'un épithélium (cellules épithéliales du plexus choroïdien [CPEC]) qui produit et sécrète le liquide céphalorachidien (CSF) dans les ventricules du cerveau (87). Comme avec la BBB, la BCSFB est constituée de jonctions

serrées inhibant la diffusion paracellulaire de molécules hydrosolubles (83). Bien que la BBB représente une plus grande surface d'invasion, des études anatomopathologiques ont démontré que le plexus choroïdien des porcs est perturbé suite à l'infection par *S. suis*, incluant la présence de cellules immunitaires dans les ventricules (88). En effet, *S. suis* peut diminuer la résistance transépithéliale des CPECs (64, 89). De plus, *S. suis* possède la capacité d'adhérer aux CPECs et de traverser la BCSFB du côté basolatéral (sang) pour se rendre au côté latéral (CSF) via l'endocytose (90). *S. suis* peut aussi induire leur mort, en partie due à la présence de la SLY (91). D'autre part, l'étude du transcriptome de CPECs infectées par *S. suis* a démontré que les gènes impliqués dans la réponse inflammatoire et dans la production de médiateurs inflammatoires sont régulés à la hausse (89, 92). Ensemble, ces interactions pourraient permettre à *S. suis* d'atteindre le CSF. Une fois dans le CSF, *S. suis* est non seulement libre d'atteindre le parenchyme cérébral, mais de s'y multiplier, étant donné la présence abondante de nutriments et l'absence générale d'immunoglobulines, de complément et de leucocytes (93).

1.6.6. Activation de la réponse inflammatoire du système nerveux central et développement de la méningite

Si *S. suis* réussi à traverser la BBB, les bactéries se retrouvera directement dans le cerveau où elle fera face aux cellules résidentes (84). Parmi ces cellules, la microglie et les astrocytes sont les cellules de l'immunité innée (94). Au contraire, une fois dans le CSF, *S. suis* va devoir interagir avec les cellules des méninges qui devront être envahies avant de pouvoir atteindre le cerveau (82).

Les dommages neurologiques et la mort neuronale associés à la méningite induite par *S. suis* sont la conséquence d'une reconnaissance de composants bactériens par les cellules immunitaires de l'hôte (42, 95). En effet, la présence de *S. suis* dans le SNC augmente l'expression transcriptionnelle de TLRs, du récepteur CD14 et de médiateurs pro-inflammatoires (CCL2) et ce, dans la plupart des régions du cerveau, incluant la BBB, le plexus choroïdien et les méninges (76). La microglie, et moindrement les astrocytes, ont été démontrés comme les sources principales de ces médiateurs (76). Bien que les composants

bactériens causant cette activation soient peu connus, des études avec un mutant non encapsulé ont démontré une augmentation de la production de médiateurs inflammatoires lors de l'infection de microglies et d'astrocytes murins, suggérant, comme lors de l'infection systémique, un rôle des composants sous-capsulaires de *S. suis* (96, 97).

1.7. Facteurs de virulence

Afin de coloniser son hôte et causer une infection menant au développement de la maladie clinique, *S. suis* a développé une multitude de facteurs de virulence (42, 45). En effet, il y a eu un nombre croissant de publications portant sur l'étude des facteurs de virulence de *S. suis* dans les dernières années (63). Toutefois, bien que plusieurs d'entre eux ont été décrits comme étant « critiques » pour la virulence de la bactérie, les méthodes expérimentales employées pour l'étude des facteurs de virulence varient énormément entre laboratoires, autant pour les études *in vitro* que pour les études *in vivo* (63). Ainsi, il existe un manque de consensus et de rigueur scientifique, particulièrement en ce qui concerne ce qu'est un facteur de virulence et comment le déterminer (63). Notamment, la définition de ce qu'est un facteur de virulence reste ouverte à interprétation, puisqu'aucun barème n'a été établi. Entre autres, l'état clinique de l'hôte duquel la souche a été isolée, le modèle d'infection expérimentale *in vivo* utilisé, les études *in vitro* effectuées vont tous affecter cette définition (63). De plus, le choix de la souche, en raison de son bagage génétique, pourrait aussi influencer le résultat (63). Il est donc important de garder en tête que *S. suis* est un microorganisme hautement complexe et qu'il est difficile de le catégoriser selon la présence ou l'absence de facteurs de virulence décrits ou proposés.

1.7.1. La capsule polysaccharidique (rôle dans la pathogenèse)

La CPS est une couche structurée de polysaccharides que certaines bactéries sont capables de synthétiser et d'exporter à leur surface. La CPS de *S. suis* est l'un des facteurs de virulence les plus importants, selon les études réalisées avec le sérotype 2 (42, 67, 98). De plus, la classification de *S. suis* en différents sérotypes est basée sur l'antigénicité des CPS due à des différences de composition et de structure (28). Les aspects relatifs aux structures des

CPS de *S. suis*, aux gènes responsables de la synthèse des CPS et à leur biosynthèse seront décrits plus tard. Ici, nous allons surtout nous attarder au rôle important joué par la CPS dans la pathogenèse des infections à *S. suis*, comme celle-ci est le seul facteur de virulence de *S. suis* considéré comme étant critique (99).

1.7.1.1. Propriétés et fonctions

Le rôle de la CPS dans la virulence de *S. suis* sérotype 2 a été extensivement étudié dans les dernières années et plusieurs fonctions ont été attribuées à sa présence lors de l'infection, dont la modulation de l'adhésion et de l'invasion, un rôle anti-phagocytaire et un rôle immunomodulateur (42, 67, 68, 98, 100), Une étude récente avec le sérotype 14 a démontré que la présence de sa CPS est tout aussi importante pour sa pathogenèse (101). Ainsi, peu d'information est actuellement disponible sur le rôle de la CPS chez les autres sérotypes de *S. suis*.

1.7.1.2. Rôle dans la modulation de l'adhésion et de l'invasion

La CPS du sérotype 2 de *S. suis* a été décrite comme pouvant moduler l'adhésion et l'invasion aux cellules de l'hôte en interférant avec l'action des adhésines (42). En effet, l'absence de la CPS favorise l'adhésion aux cellules épithéliales et endothéliales et favorise également l'invasion des cellules épithéliales du larynx humain et des BMECs porcines (85, 102-104). Ainsi, étant donné que l'adhésion aux cellules figure parmi les premières interactions hôte-pathogène et qu'elle est cruciale à la colonisation, l'expression de la CPS pourrait nuire à celle-ci (45). Tel que mentionné précédemment, *S. suis* pourrait moduler à la baisse l'expression de sa CPS afin de favoriser le contact entre ses adhésines à la surface cellulaire lors de la colonisation (45).

1.7.1.3. Propriétés anti-phagocytaires

La CPS est d'abord et avant tout un facteur anti-phagocytaire qui permet aux sérotypes 2 et 14 d'éviter les premières lignes de défense immunitaire de l'hôte, c'est-à-dire les cellules

phagocytaires de la réponse immunitaire innée, dont les macrophages, les DCs et les neutrophiles (42, 101). En effet, la CPS du sérotype 2 inhibe spécifiquement la transduction des signaux intracellulaires nécessaires à la phagocytose chez les macrophages (72). De plus, lorsque *S. suis* entre en contact avec les macrophages, sa CPS permet de déstabiliser les microdomaines lipidiques, ce qui prévient sa reconnaissance et sa phagocytose (105). La présence de CPS interfère également dans la phagocytose et le « killing » par les DCs murines et porcines et par les neutrophiles porcins (65, 66, 106, 107). Cette propriété que confère la présence de la CPS, du moins aux sérotypes 2 et 14, est cruciale pour leur virulence. En son absence, *S. suis* est incapable de résister au « killing » par les leucocytes sanguins et est rapidement éliminé de la circulation sanguine (67, 68). De plus, la présence de la CPS du sérotype 2 interfère dans le dépôt du complément à la surface bactérienne, prévenant l'opsonophagocytose en absence d'anticorps spécifiques (65, 66, 107).

Bien que les études sur le rôle de la CPS se sont concentrées sur les souches du sérotype 2, et moindrement sur le sérotype 14, une étude *in vitro* a démontré qu'une souche de sérotype 1 est moins internalisée par les DCs humaines dérivées de monocytes que des souches des sérotypes 2, 7 et 14, alors que les souches pour les sérotypes 4 et 9 sont plus internalisées (108). Ainsi, il est possible que la composition de la CPS puisse influencer les propriétés anti-phagocytaires de celle-ci. Cependant, d'autres facteurs tels que l'épaisseur de la CPS et le bagage génétique (génome et protéome) pourraient également influencer les propriétés anti-phagocytaires des souches à l'étude (108).

1.7.1.4. Immunomodulation

La CPS participe également dans la modulation de la réponse inflammatoire (42). En effet, la présence de CPS permet de masquer la surface bactérienne, incluant les composés sous-capsulaires ayant des propriétés immunostimulatrices (42). Des tests *in vitro* avec différents types cellulaires, dont les DCs et les macrophages, ont permis de démontrer qu'un mutant non encapsulé de *S. suis* sérotype 2 induit davantage l'expression de cytokines pro-inflammatoires comparativement à la souche sauvage encapsulée, telles que le TNF, l'IL-6, I'IL-12p70 et le CXCL1 (73, 107, 109). Cependant, aucune information n'était disponible au

début de ces travaux sur la capacité des CPS de *S. suis* à influencer directement la réponse immunitaire, soit par immunostimulation ou immunomodulation,

1.7.2. Les marqueurs de virulence classiques

1.7.2.1. La suilysine

S. suis sécrète une panoplie de composés, dont une toxine hémolytique nommée suilysine. Avec une taille de 54 kDa, celle-ci appartient au groupe de toxines cytolytiques liant le cholestérol de la membrane cellulaire eucaryote (110). Cette toxine n'est cependant pas retrouvée chez tous les sérotypes de *S. suis*. La SLY est similaire à la pneumolysine de *S. pneumoniae*, à l'exception que cette dernière n'est pas sécrétée contrairement à la toxine de *S. suis*. La SLY se rapproche également de la streptolysine O du streptocoque du groupe A, la listeriolysine de *L. monocytogenes* et la perfringolysine *de Clostridium perfringens* (111). Ces toxines, de même que la SLY, sont caractérisées par une perte d'activité et un regain de celle-ci, suite à l'oxydation et à la réduction, respectivement. De plus, elles sont inhibées par de faibles quantités de cholestérol et sont responsables de la formation de pores transmembranaires (112, 113).

Tout comme n'importe quel autre facteur de virulence putatif, le rôle de la SLY dans la pathogenèse de l'infection causée par *S. suis* a été investigué, mais reste tout de même nébuleux (114). La SLY est toxique pour les cellules endothéliales et épithéliales de même que pour les neutrophiles, les monocytes et les macrophages (65, 71, 104, 115, 116). De plus, la SLY induit la production de médiateurs pro-inflammatoires par les BMECs humaines et porcines, les cellules mononuclées du sang porcin, les macrophages alvéolaires porcins et les DCs murines (85, 107, 117-120). Elle induit également une régulation à la hausse des molécules adhésives des monocytes humains et est impliquée dans la sécrétion de l'acide arachidonique, précurseur des prostaglandines et des leukotriènes, par les cellules endothéliales humaines (121, 122). Finalement, la SLY contribue à la résistance contre l'opsonophagocytose en l'absence d'anticorps spécifiques par les DCs murines (107).

In vivo, le rôle de cette toxine reste controversé. En effet, aucune mortalité n'a été observée chez des souris infectées avec le surnageant d'une culture d'une souche sécrétant de la SLY (123). Toutefois, un mutant déficient pour la SLY est avirulent dans un modèle murin, mais ne démontre qu'une virulence légèrement réduite lors de l'infection systémique chez le porc (123). Enfin, lors d'une étude de challenge chez le porc, la souche mutante a induit une maladie similaire à celle de la souche-mère (120). Il est donc difficile de considérer cette toxine en tant que facteur de virulence critique, compte tenu de la variation obtenue dans les différentes études.

1.7.2.2. Le facteur extracellulaire et la « muraminidase-released protein »

Ces deux protéines ont été parmi les premiers facteurs de virulence putatifs décrits pour *S. suis* sérotype 2 (124). La « muraminidase-released protein » est une protéine de 136 kDa ancrée dans la paroi cellulaire par un motif LPXTG, mais qui peut être relâchée dans le surnageant lors de la croissance bactérienne (9, 124). Au contraire, le facteur extracellulaire, codé par le gène *epf*, est une protéine de 110 kDa présente seulement dans le surnageant des cultures bactériennes (9, 124). Les souches de *S. suis* sérotype 2 de phénotype MRP+EF+ sont associées à des infections plus sévères chez le porc (125, 126). Par contre, lorsque des mutants isogéniques pour MRP et EF ont été inoculés chez des porcelets, leur virulence était égale à celle des souches-mères (127). Une étude récente a démontré que parmi les souches nord-américaines de *S. suis* sérotype 2, les phénotypes les plus communs sont MRP−EF−SLY− à 44% et MRP+EF−SLY− à 51%, correspondant aux ST25 et ST28, respectivement (39). Cependant, étant donné que les rôles spécifiques de la MRP et EF dans la pathogenèse de *S. suis* n'ont pas été clarifiés, ces protéines devraient être seulement considérées comme étant des marqueurs associés à la virulence (39).

1.7.3. Autres facteurs

En plus des facteurs décrits plus haut, plusieurs centaines d'autres facteurs de virulence confirmés ou putatifs ont été décrits pour *S. suis*, en particulier pour le sérotype 2 (42, 45, 63). La **Figure 1** ci-dessous résume ces différents facteurs selon les fonctions biologiques

rapportées pour la plupart de ceux-ci, mais elle ne se veut pas exhaustive. Parmi tous ces facteurs rapportés, une grande majorité n'ont pas été caractérisés à l'aide de méthodes appropriées pour clairement établir leur rôle dans la pathogenèse de *S. suis* (99). À titre d'exemple, pour tous les facteurs impliqués dans le métabolisme bactérien, plusieurs études ne font pas la distinction entre le « fitness » bactérien et un potentiel rôle dans la virulence. Un second exemple est que plusieurs de ces facteurs n'ont été rapportés que pour une seule souche, et le degré de conservation parmi toutes les souches de *S. suis* d'un facteur demeure inconnu.

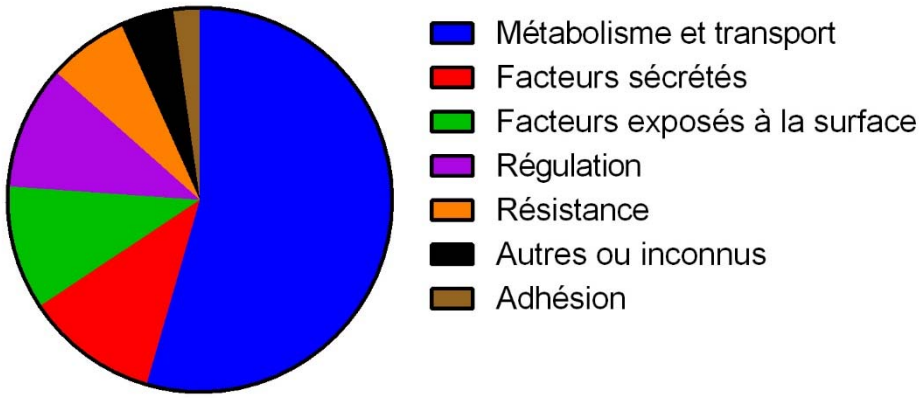


Figure 1. Principales fonctions biologiques rapportées pour les autres facteurs de virulence de *S. suis*.

Préparée à partir des données de (99).

1.8. Approches thérapeutiques et prophylactiques

1.8.1. Antibiothérapie

À ce jour, les antibiotiques restent le premier choix pour traiter les infections à *S. suis* (128). Toutefois, le choix de l'antibiotique doit se faire selon la susceptibilité de la souche responsable de l'infection, tout en tenant compte des résistances rapportées dans la filière porcine suite à la surutilisation des antibiotiques (128, 129). Néanmoins, l'ampicilline, le

ceftiofur, la tiamuline, le triméthoprime et les sulfonamides restent de bons choix pour le traitement de la maladie (3). Il est important de se rappeller que *S. suis* ne pourra jamais être complètement éliminé d'un animal colonisé (128). D'autre part, lorsque les animaux présentent des signes de méningites, l'administration d'un anti-inflammatoire en combinaison avec l'antibiotique choisi pourrait améliorer la réponse de l'animal à son traitement (3).

Les antibiotiques étaient utilisés autrefois pour la prophylaxie, c'est-à-dire traiter, via la nourriture ou l'eau par exemple, les animaux sains d'un parc ou d'un troupeau pour prévenir l'apparition de la maladie lorsque des signes cliniques étaient observables chez certains individus (3). Également, bien que non spécifique à *S. suis*, les antibiotiques utilisés à des doses sous-thérapeutiques aux fins de promotion de la croissance au sein de troupeaux sains agissent par l'élimination d'infections sous-cliniques et ainsi contribuer au mieux-être des animaux et au gain de poids (130). Cependant, suite à l'association de ces pratiques avec l'émergence et la propagation de l'antibiorésistance, et à la volonté de bien utiliser les antibiotiques seulement lorsque nécessaire, ces pratiques ont soit déjà été interdites (comme en Europe) ou sont en voie de l'être bientôt partout ailleurs (131, 132).

Pour ce qui est des infections chez les humains, et spécialement lors de méningites, une antibiothérapie agressive est habituellement recommandée (8). On parle alors de traitements avec de fortes doses pour les cas sévères, en combinant la pénicilline G avec un second antibiotique comme la ceftriaxone, la gentamicine, le chloramphénicol et l'ampicilline, administrés par voie intraveinale pour une période de 10 à 14 jours (13). Toutefois, l'utilisation d'antibiotiques a un effet limité chez l'humain lors du choc septique (8).

1.8.2. Vaccination

Comme il n'existe présentement aucun vaccin contre *S. suis* commercial disponible pour usage en médecine humaine ou vétérinaire, la prévention de la transmission aux humains dépend d'abord et avant tout du contrôle de la maladie chez le porc (8). Des mesures de santé publique accompagnées de campagnes de sensibilisation, concernant notamment les mesures

d'hygiène ou les dangers de certains plats traditionnels (comme le Tiet Canh au Vietnam, mets à base de sang cru de porc), permettront de réduire le nombre d'infections humaines (8).

Afin de prévenir le développement de maladies causées par *S. suis* chez les animaux, la vaccination est la mesure la plus recommandée. Toutefois, il existe très peu d'information concernant son efficacité et peu de fermes au Canada utilisent un vaccin (133). De plus, le plus grand problème auquel font face les études de vaccination est la faible immunogénicité de *S. suis* (133). Entre autre, la CPS, faiblement immunogène, masque les composants sous-capsulaires qui eux le sont (134, 135). De plus, *S. suis* a développé plusieurs mécanismes pour déjouer les différentes étapes de la réponse adaptative. En effet, il a récemment été démontré que *S. suis*, en partie par l'entremise de sa CPS, interfère dans la présentation antigénique par les DCs, que ce soit en retardant/compromettant l'expression du complexe majeur d'histocompatibilité de classe II (CMH-II) et/ou en interférant dans la production d'interleukine (IL)-12p70, impliquée dans la différenciation des lymphocytes T $CD4^+$ (136). Ceci a pour conséquence une activation sous-optimale des lymphocytes T, des lymphocytes B et de la réponse adaptative globalement (133, 135, 137). Néanmoins, différents types de vaccins expérimentaux ont été testés au cours des dernières années (133).

1.8.2.1. Bactérines

Les bactérines sont généralement le premier choix et sont présentement les seuls vaccins utilisés sur le terrain (133). Elles sont constituées de la bactérie entière inactivée, soit par traitement à la chaleur (rare) ou au formaldéhyde (très courant) (133). Une alternative à la possible altération des épitopes protecteurs lors de l'inactivation au formaldéhyde serait l'inactivation par traitement avec un antibiotique (138). Néanmoins, l'efficacité des bactérines demeure controversée, notamment par le faible nombre d'études sur le terrain qui ont été rapportées, mais également par les erreurs au niveau de la conception de ces études qui ne permettent pas d'évaluer de manière appropriée la protection (3, 133). Une utilisation populaire des bactérines est dans la création de vaccins autogènes qui sont préparés à partir d'une souche isolée au sein d'un troupeau donné pour être administré exclusivement au troupeau d'origine (133).

1.8.2.2. Vaccins sous-unitaires protéiques

Outre les bactérines, l'approche vaccinale étudiée est l'utilisation de vaccins sous-unitaires de la bactérie, en particulier composés de protéines purifiées. Ceux-ci sont cependant encore tous au stade expérimental. Dans ce type de vaccin, l'antigène choisi doit idéalement être présent chez plusieurs souches et/ou sérotypes de *S. suis* afin de conférer une protection « universelle » contre le pathogène (133). Bien qu'un grand nombre de protéines ont été étudiées, ces vaccins ne sont pas encore commercialement disponibles (133).

Parmi ces protéines candidates, nous retrouvons les trois principaux marqueurs de virulence de *S. suis*, soit la SLY, l'EF et la MRP. Ces trois protéines ont déjà été démontrées comme étant hautement immunogènes dans plusieurs études employant divers modèles animaux et protocoles d'immunisation (133). En plus du sérotype 2, la protéine MRP a également été détectée chez des souches pour les sérotypes 1, 1/2, 3, 4, 5, 7, 8, 9, 10, 12, 14, 15, 16, 18, 21, 22, 26 and 28 (133). De plus, des souches produisant l'EF ont été identifiées chez les sérotypes 1, 2, 1/2, 14 et 15 (133). Également, la SLY est présente dans la majorité des souches virulentes des sérotypes 2 et 9 en Europe, et a également été détectée chez les sérotypes 1, 1/2, 3, 4, 5, 7, 8, 10, 12, 14, 15, 18, 19, 23 et 30 (133). Vu la grande distribution parmi les sérotypes *S. suis* de MRP et SLY, et la distribution plus limitée d'EF, il a été suggéré que ces protéines peuvent servir d'antigènes avec un large spectre de protection. Plusieurs études réalisées avec ces protéines ont produit des résultats contradictoires, avec une influence importante du choix de l'adjuvant et un manque d'association entre protection et la présence d'anticorps spécifiques (133). Ainsi, la production de hauts titres d'anticorps contre MRP, EF et/ou SLY ne garantit pas la protection, ce qui limite leur application comme vaccins sous-unitaires.

Esgleas et al. (139) ont identifié en 2008 une nouvelle protéine de surface de 52 kDa liant la fibronectine comme une énolase exprimée par tous les sérotypes de *S. suis*. En plus d'être une protéine hautement conservée, la détection d'anticorps anti-énolase dans des sérums

de porcs convalescents font de cette protéine un excellent candidat pour un vaccin universel contre les infections à *S. suis* (133). Malheureusement, une étude chez la souris (avec Quil-A® comme adjuvant) n'a pas mené à la protection, malgré une forte réponse IgG, de même qu'une immunisation passive avec un sérum hyperimmun de lapin anti-énolase n'a pas conféré de protection (140). D'autres études employant l'adjuvant complet de Freund (FCA) ont permis de démontrer la protection conférée par l'énolase chez la souris (141, 142). Ainsi, malgré son grand potentiel comme antigène universel, des études additionnelles avec l'énolase en évaluant différents adjuvants et la protection chez le porc sont requises.

Li et al. (143) ont identifié en 2006 une protéine de 110 kDa qu'ils ont nommé « surface antigen one » (Sao) comme une protéine membranaire de surface et réagissant avec des sérums de porcs convalescents. Sao a également été détectée parmi les sérotypes 1, 2, 1/2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 17, 18, 19, 21, 23, 25, 26, 27, 28, 29, 30, 31 et 33. Lors d'une immunisation réalisée chez la souris (avec Emulsigen-Plus® comme adjuvant), Sao a été démontrée comme étant hautement immunogène, mais cependant cette réponse n'était pas protectrice. Lors d'une seconde étude réalisée chez la souris et chez le porc, avec le Quil-A® comme adjuvant cette fois-ci, l'immunisation de Sao a conféré une protection (144). Des résultats similaires ont également été obtenus par d'autres, en plus de démontrer une protection croisée contre des sérotypes autres que le 2 (141, 145-147). Contrairement aux protéines mentionnées précédemment, Sao n'est pas un facteur de virulence critique, ce qui illustre le fait qu'antigènes protecteurs et facteurs de virulence ne sont pas nécessairement synonymes (148).

1.8.2.3. Vaccins sous-unitaires à base de CPS

Un vaccin basé sur la CPS de *S. suis* offre de nombreux avantages : d'abord, la CPS étant la couche la plus externe de l'enveloppe bactérienne, les anticorps la ciblant sont connus pour être hautement opsonisants et protecteurs, tel que déjà démontré pour plusieurs pathogènes encapsulés d'importance en médecine humaine, tels que *S. pneumoniae*, GBS, *Neisseria meningitidis* et *Haemophilus influenzae* (149-151). De plus, les anticorps dirigés

contre la CPS offrent une protection pan-sérotype, car toutes les souches encapsulées d'un même sérotype expriment la même CPS, indépendamment de la variation génotypique et phénotypique parmi ces souches, ce qui est particulièrement vrai dans le cas du sérotype 2 de *S. suis* (133). Au commencement de ce projet de doctorat, aucun vaccin de ce genre n'a encore été rapporté pour *S. suis*. Cependant, compte tenu du fait que les polysaccharides sont reconnus comme étant très pauvrement immunogènes (voir plus loin), le développement d'un vaccin de ce type comporte des défis de taille.

2. La capsule polysaccharidique (structure, locus capsulaires et biosynthèse)

Les bactéries produisent différents types de polysaccharides qui sont exprimés à leur surface sous forme de glycoconjugués (glycoprotéines et glycolipides), mais qui peuvent aussi être sécrétés (peptidoglycane, lipopolysaccharide, acides téichoïque et lipotéichoïque, exopolysaccharides, etc.) Certaines bactéries peuvent également synthétiser un réseau structuré de polymères rigide et hydraté constituant la couche la plus externe du pathogène, la CPS (152). Les CPS sont des molécules de haut poids moléculaire (de 100 à 1000 kDa) qui sont formées d'unités répétitives composées d'un à huit monosaccharides et pouvant être linéaires ou branchées. En plus des différences compositionelles (différents monosaccharides présents), la très grande diversité structurale des polysaccharides provient de la position des liens glycosidiques entre les sucres, de la forme énantiomérique des sucres (D ou L), le nombre de carbones formant la structure cyclique du sucre (forme furanose ou pyranose) ou encore la configuration du centre anomérique de chaque sucre (α ou β). De plus, les CPS peuvent être neutres ou chargées négativement, bien que quelques-unes soit dites zwitterioniques, car elles contiennent à la fois des charges négatives et positives (comme la CPS de *Bacteroides fragilis* type A, celle de *S. pneumoniae* sérotype 1, et celles des sérotypes 5 et 8 de *Staphylococcus aureus*) (153).

Comme décrit dans la section précédente, la CPS de *S. suis* joue des rôles cruciaux dans la pathogenèse de l'infection causée par *S. suis*. *S. suis* est classifié en 35 différents sérotypes capsulaires qui se distinguent par différentes compositions et structures. Ici, nous allons aborder les structures de CPS connues et les locus capsulaires décrits pour *S. suis*, la biosynthèse des CPS chez les bactéries à Gram positif, ainsi que les structures de CPS rapportées chez d'autres streptocoques.

2.1 Composition et structure de la CPS du sérotype 2 de *S. suis*

Au commencement de ce projet de doctorat, seule la structure de la CPS du sérotype 2 était connue, telle que rapportée en 2010 par Van Calsteren et al. (154). Celle-ci est composée de galactose (Gal), de glucose (Glc), de *N*-acétylglucosamine (GlcNAc), de rhamnose (Rha) et de 'acide sialique (Neu5Ac). La séquence répétitive de l'unité répétitive du sérotype 2 est présentée à la **Figure 2**. On peut remarquer la présence d'un acide sialique lié au galactose par un lien α2-6.

CPS de *S. suis* sérotype 2

A

α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc α-D-Gal*p*

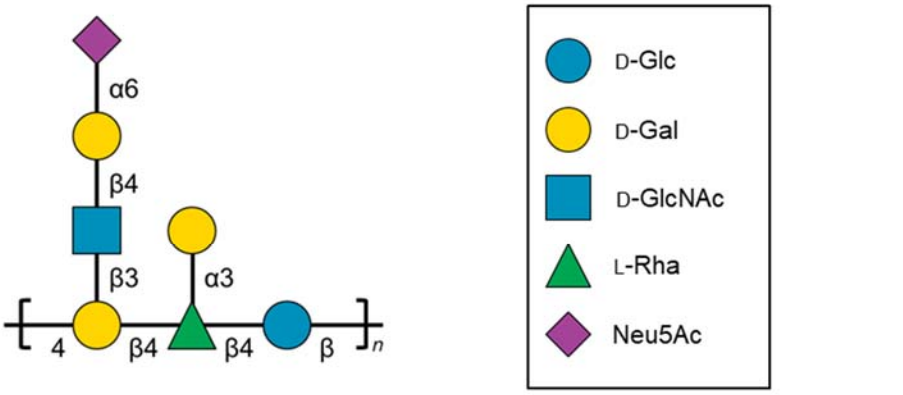
1 1

↓ ↓

3 3

[4)-β-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→]$_n$

B



C

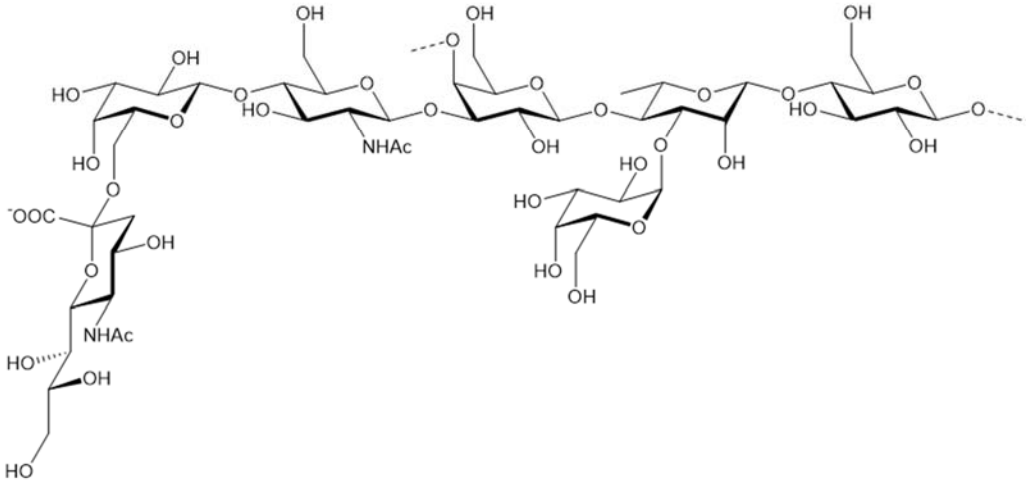


Figure 2. Structure de l'unité répétitive de la CPS du sérotype 2 de *S. suis* telle que décrite par Van Calsteren et al. (154).

(A) Représentation textuelle. (B) Représentation symbolique selon le système de « Symbol Nomenclature for Glycans » tel qu'établi par Varki et collaborateurs (155). (C) Représentation sous forme de chaise.

2.2. Locus capsulaires

Le *locus* de la CPS de *S. suis* a été caractérisé en premier chez le sérotype 2 par Smith et al. en 1999 (156). Il a fallu cependant attendre en 2013 afin que l'ensemble des différents locus capsulaires correspondant aux 35 sérotypes de *S. suis* soient caractérisés par Okura et al. (157).

2.2.1. Organisation génétique du *locus* capsulaire

Il est proposé que les gènes présents dans le *locus* de la CPS soient transcrits sous un même acide ribonucléique (ARN) polycistronique et régulés sous un seul même promoteur situé en amont du gène *cpsA* (**Annexes, ARTICLE XIII**). La taille des locus de la CPS varie en fonction du sérotype. Les gènes codants pour la biosynthèse, l'exportation et la polymérisation de la CPS se retrouvent chez la plupart des sérotypes entre les gènes *orfZ* et *aroA*. Néanmoins, pour d'autres sérotypes, le *locus* chromosomique codant pour les gènes de la CPS se retrouve ailleurs dans le génome. Basé sur les différents emplacements du locus de la CPS dans le génome, cinq grands patrons de locus chromosomiques ont été observés à partir des 35 sérotypes, soit les patrons Ia, Ib, II, III et IV. Le patron le plus commun est le patron Ia comprenant notamment les sérotypes 1, 1/2, 2, 3, 7, 8, 14 et 18. Le second patron en importance est le patron Ib, situé entre *orfZ* et *glf*, et qui comprend notamment le sérotype 9 (157).

Malgré que le *locus* de la CPS varie entre les différents patrons chromosomiques de locus capsulaires, il existe par contre des caractéristiques communes parmi plusieurs sérotypes. Ainsi, on retrouve les gènes de régulation de la biosynthèse de la CPS, *cpsA*, *cpsB*, *cpsC* et *cpsD* chez tous les sérotypes. Les gènes codant pour des polymérases (Wzy), flippases (Wzx) et différentes glycosyltransférases sont également retrouvés chez l'ensemble des sérotypes. Ceux-ci comprennent également un gène codant pour la transférase initiale (157).

Une caractéristique importante est la présence chez certains sérotypes de gènes associés à la biosynthèse et au transfert de sucres uniques, comme par exemple l'acide sialique du sérotype 2. Ces gènes, soit *neuBCDA* (synthèse) et *cpsN* (sialyltransférase) ont été caractérisés chez les sérotypes 1, 1/2, 2, 6, 13, 14, 16 et 27. Curieusement, les gènes codant pour la biosynthèse de l'acide sialique chez le sérotype 13 sont phylogénétiquement différents des autres sérotypes sialylés mentionnés (157). De plus, malgré la détection des gènes responsables pour la biosynthèse de l'acide sialique, une étude précédente par Charland et al. (158) employant la lectine de *Sambucus nigra* (SNA; spécifique pour les Neu5Ac-α2,6-Gal/GalNAc- (159)) a permis de démontrer la présence d'acide sialique seulement chez les souches des sérotypes 1, 1/2, 2, 14, 15 et 16 de *S. suis*. Ainsi, on connait avec quasi-certitude que les sérotypes 1, 1/2, 2, 14 et 16 possèdent un acide sialique lié en α2,6- dans leur CPS (confirmé dans le cas du sérotype 2 (154)). Dans le cas du sérotype 15, positif avec la SNA, mais ne possédant pas de gènes, cette réaction demeure à être expliquée : une possibilité est que cette lectine peut reconnaître (plus faiblement) le Gal/GalNAc en absence de Neu5Ac dans certaines situations (134, 159). Pour ce qui est des sérotypes possédant les gènes, mais qui sont négatifs avec la SNA, soit les sérotypes 13 et 27, il demeure à valider si ces sérotypes possèdent l'acide sialique au sein de leurs CPS respectives.

Également, la plupart des sérotypes possèdent un gène unique ou sérotype-spécifique. Les exceptions sont les sérotypes 1, 1/2, 2 et 14 où aucune polymérase, glycosyltransférase ou flippase unique n'a pu être trouvée. Les *locus* des CPS pour les sérotypes 1 et 14 et les sérotypes 2 et 1/2 sont pratiquement identiques, d'où l'absence de gène unique. Par le séquençage et l'assemblage de nouveaux génomes pour chacun de ces sérotypes, il a été déterminé que les sérotypes 2 et 14 possèdent un nucléotide G à la position 483 du gène *cpsK* (codant pour la galactosyltransférase) alors que les sérotypes 1 et 1/2 ont un C ou un T à cette même position nucléotidique. Cette mutation ponctuelle se traduit en un tryptophane à la position 161 de la protéine CpsK chez les sérotypes 2 et 14, comparativement à une cystéine pour les sérotypes 1 et 1/2. Ceci permet d'expliquer pourquoi ces deux paires de sérotypes ne peuvent être résolues lors de la sérotypie par « polymerase chain reaction » (PCR).

2.2.2. Nouveaux locus capsulaires (NCLs) et nouveaux sérotypes potentiels

Récemment, Zhenghan et al. (21) ont décrit les locus capsulaires de 179 souches de *S. suis* isolées des porcs sains en Chine et identifiées comme non typables par les tests sérologiques et moléculaires. Ils ont pu ainsi classer 44% (78/179) des isolats non typables en huit nouveaux locus capsulaires (identifiés comme « novel capsular *loci* » ou NCL1 à 8). Curieusement, chacun des huit *locus* se distingue par la présence d'un gène codant pour une polymérase spécifique et pourrait conséquemment représenter de nouveaux sérotypes. Cependant, il a été démontré que 25 des 79 isolats identifiés comme appartenant à un nouveau NCL sont non encapsulés en raison de mutations dans le *locus* capsulaire, rendant ainsi impossible le sérotypage de ces souches par de futurs tests sérologiques (21).

Parallèlement, Pan et al. (160) ont également caractérisé une souche non typable isolée d'une épidémie de *S. suis* chez des porcelets en Chine et qui pourrait appartenir à un nouveau sérotype. Ils ont démontré que la souche CZ130302, non typable par tests sérologiques et moléculaires, possédait une polymérase unique. Il est proposé que ce nouveau sérotype se nomme Chz (160).

Depuis, huit autres nouveaux locus capsulaires potentiels ont été caractérisés chez *S. suis,* portant le nombre total de NCLs à 17 (1–16 et Chz). À l'opposé des premiers NCLs (1–8), ces derniers NCLs ont été caractérisés à partir de souches de *S. suis* isolées de porcs sains majoritairement, mais également de porcs malades. Par contre, tous les NCLs caractérisés (y compris Chz) ont été découverts chez des souches isolées de porcs en Chine, et représentent potentiellement des nouveaux types capsulaires endémiques. Une statistique intéressante de cette dernière étude est que 94.8% des « vraies » souches non sérotypables de cette étude appartiennent à l'un des 17 NCLs caractérisés (161). On considère une « vraie » souche non typable lorsqu'elle possède un *locus* capsulaire et que celui-ci ne correspond pas à un *locus* capsulaire d'un sérotype déjà décrit. Toutefois, ces nouveaux sérotypes potentiels ne sont pas

encore reconnus officiellement et ne sont présentement pas considérés dans les laboratoires diagnostiques de *S. suis*.

2.2.3. Mutants de la CPS chez *S. suis*

Les premiers mutants de la CPS chez *S. suis* ont été obtenus par insertion de transposon (100). Une seule insertion était suffisante afin d'inhiber la biosynthèse de la CPS, ce qui a eu pour conséquence sa perte et un phénotype non encapsulé (100). De plus, ces travaux ont démontré que l'absence de CPS modifie l'hydrophobicité à la surface bactérienne, diminue la résistance à la phagocytose par les phagocytes murins et porcins et favorise l'élimination rapide de la bactérie du sang (100).

Depuis, plusieurs autres mutants de la CPS ont été obtenus, mais tous chez le sérotype 2. En effet, les rôles de la protéine régulatrice CpsB (67), de la transférase initiale CpsE (67), de différentes glycosyltransférases (CpsF, CpsG, CpsJ et CpsL) (107, 162) ainsi que de NeuB et NeuC, enzymes impliquées dans la biosynthèse de l'acide sialique (**Annexes, ARTICLE XIV**) (102), ont été décrits.. Peu importe la délétion, un phénotype non encapsulé est toujours obtenu. Plus récemment, un mutant de la CPS a été obtenu pour le sérotype 14 (CpsB) (101). Toutefois, malgré l'importance des autres sérotypes de *S. suis*, aucun mutant d'un sérotype autre que les sérotypes 2 et 14 n'a encore été étudié.

2.3. Biosynthèse de la CPS chez les bactéries à Gram positif

Il existe trois différentes voies de synthèse et de production de la CPS chez les bactéries; la voie *wzy*, la voie de la synthase et la voie du transporteur ABC. La voie du transporteur ABC, nommée selon le mécanisme utilisé pour exporter la CPS à l'extérieur, existe uniquement chez les bactéries à Gram négatif; pour cette raison, celle-ci ne sera pas décrite davantage. Les bactéries à Gram positif utilisent quant à elles la voie Wzy et la voie de la synthase. La présence de plusieurs enzymes clés est une caractéristique importante de la voie Wzy : les glycosyltransférases, la polymérase (Wzy) et la flippase (Wzx). La voie de la synthase requiert seulement une enzyme pour synthétiser et exporter la CPS vers l'extérieur. Règle générale, une espèce bactérienne va utiliser l'une ou l'autre des voies de biosynthèse de la CPS. Une exception intéressante est chez *S. pneumoniae*, où la majorité des sérotypes utilisent la voie Wzy, alors que certains, notamment les sérotypes 3 et 37, utilisent la voie de la synthase (163). Basé sur la présence de gènes codant les différentes enzymes des locus capsulaires des différents sérotypes de *S. suis*, c'est-à-dire les glycosyltransférases, les polymérases et les flippases, il est assumé que la biosynthèse de la CPS de *S. suis* procède via la voie de la polymérase (Wzy) pour ses 35 sérotypes (157).

2.3.1. La voie Wzy

La voie Wzy se déroule selon quatres étapes; (i) l'initiation, (ii) la synthèse de l'unité répétitive, (iii) l'exportation et (iv), la polymérisation et subséquement (v) la translocation au peptidoglycane **(Figure 3)** (163).

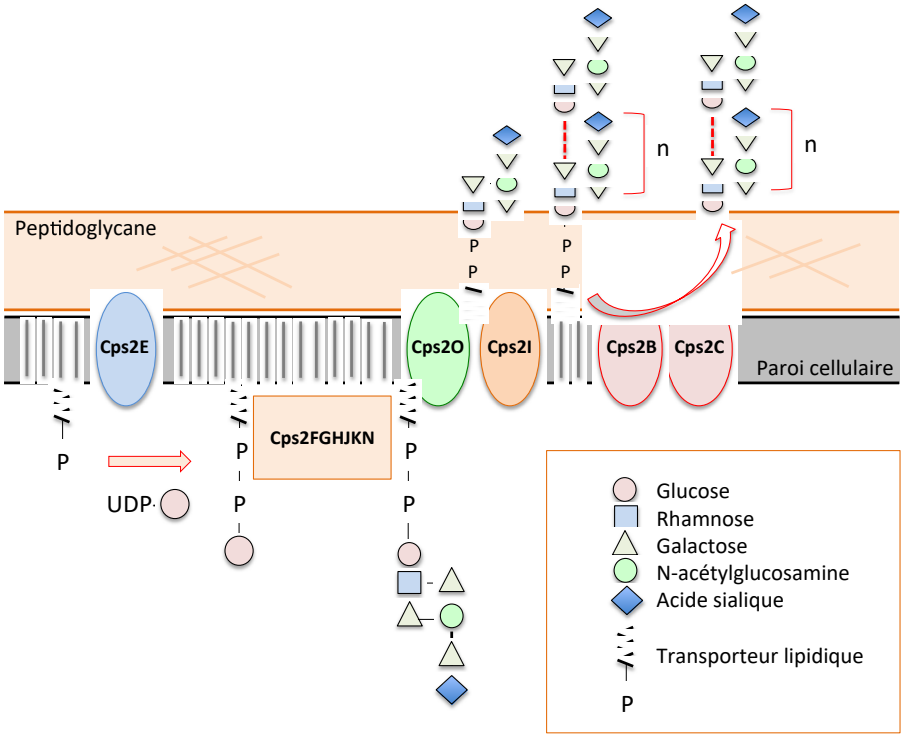


Figure 3. Biosynthèse de la CPS polysaccharidique du sérotype 2 de *S. suis* par la voie de synthèse Wzy.

Les deux enzymes clées sont la polymérase Wzy (Cps2I) et la flippase Wzx (Cps2O). L'initiation de la biosynthèse de la CPS débute avec le transfert d'un sucre-1-phosphate au précurseur lipidique (Und-P), le même accepteur qui est utilisé dans la synthèse du peptidoglycane et l'acide téichoïque chez les bactéries à Gram positif. Après initiation, les sucres subséquents sont ajoutés par les différentes glycosyltransférases encodées dans le locus de la CPS. Il y a ensuite exportation de la CPS de l'autre côté de la paroi par la flippase (Wzx). La polymérase (Wzy) effectue la polymérisation en chaînes polysaccharidiques. Le complexe Wzd/Wze (codé par cpsB/cpsC) transfère ensuite la CPS mature au peptidoglycane pour l'y ancrer. Dans les bactéries à Gram positif, la majorité des polymères restent associés à la bactérie par un lien covalent au peptidoglycane ou à des composants membranaires. Figure inspirée de Lakkitjaroan et al. (164).

(**i**) La biosynthèse de la CPS commence par le transfert d'un sucre-1-phosphate à un transporteur lipidique, le undécaprényl-phosphate (Und-P). Ce dernier, dont la disponibilité est limitée, joue un rôle central chez la bactérie puisqu'il est également impliqué dans d'autres fonctions biologiques essentielles chez les bactéries à Gram positif, notamment dans la biosynthèse du peptidoglycane et de l'acide téichoïque. La voie de biosynthèse de la CPS compétitionne donc avec d'autres voies métaboliques de la bactérie. Le transfert du premier

sucre au transporteur lipidique est assuré par une protéine membranaire, la transférase initiale, qui est codée par *cpsE* chez la plupart des sérotypes (163).

(**ii**) La deuxième étape consiste en l'ajout de différents sucres subséquents par les glycosyltransférases codés par différents gènes au sein du locus de la CPS. La nature des glycosyltransférases varie en fonction du sérotype et dicte ainsi la composition et la structure de la CPS (163).

(**iii et iv**) Les sous-unités sont ensuite exportées à la surface de la paroi membranaire par la flippase (Wzx). Elles sont ensuite polymérisées en chaînes polysaccharidiques par la polymérase Wzy. La polymérase chez *S. suis* est une enzyme sérotype-spécifique et reconnaît une structure oligosaccharidique précise (163).

(**v**) Le complexe Wzd/Wze (codé par *cpsB*/*cpsC*) transfère ensuite la CPS formée au peptidoglycane. Dans les bactéries à Gram positif, la majorité des polymères restent associés à la bactérie par un lien covalent au peptidoglycane ou à des composants membranaires (164).

De plus, le *locus* de la CPS code pour un système phosphorégulateur, composé d'une tyrosine kinase et d'une tyrosine phosphatase, permettant le contrôle sur la longueur des chaînes polysaccharidiques. Ce système est codé par quatre gènes dits « régulateurs » : *cpsA* (Protéine régulatrice membranaire), *cpsB* (Protéine régulatrice de la longueur des chaînes), *cpsC* (Tyrosine-protéine kinase) et *cpsD* (Protéine-tyrosine phosphatase) (157).

2.4. Structures et fonctions des CPS du streptocoque du groupe B

GBS, nommé selon la classification des streptocoques selon Lancefield, est une bactérie à Gram positif commensale de la flore vaginale et représente la cause la plus commune d'infection invasive néonatale. GBS cause méningites, sepsis, pneumonies et arthrites. De plus, les infections invasives causées par GBS sont de plus en plus prévalentes chez les personnes âgées de 65 ans et plus (165).

La CPS de GBS est également l'unité à la base du sérotypage et permet la distinction de 10 types différents : soit Ia, Ib, et II–IX (**Figure 4**). Malgré la similarité de structure et de composition entre plusieurs sérotypes, notamment entre les types Ia et III, aucune réaction croisée n'a été rapportée chez GBS (166, 167).

À l'instar de *S. suis*, GBS utilise la voie de la polymérase (Wzy) pour exprimer sa CPS. Les structures de l'ensemble des dix différentes CPS de GBS ont été caractérisées et possèdent toutes un acide sialique en position terminale. Comme mentionné, une différence importante est la nature du lien de l'acide sialique relié au galactose au sein de la CPS entre *S. suis* (α2,6-Gal) et GBS (α2,3-Gal) (168). Étant donné la présence de l'acide sialique dans tous les types de CPS chez GBS, il est avancé que son acide sialique (α2,3-) est un élément clé dans l'évasion des défenses immunitaires de l'hôte (167). De plus, toutes ces CPSs possèdent une charge négative conférée par l'acide sialique.

Malgré la similarité de composition et de structure avec celle du sérotype 2 de *S. suis*, la CPS de GBS ne semble pas être un facteur anti-phagocytaire aussi important que chez *S. suis*. En effet, il a été démontré que GBS type III est facilement phagocyté par les DCs et les macrophages. GBS se caractérise par une meilleure survie intracellulaire (comparativement à *S. suis*) et la CPS semble nécessaire, au moins en partie, à ces propriétés. Il est suggéré que GBS est davantage un pathogène intracellulaire transitoire, alors que *S. suis* est un pathogène strictement extracellulaire. D'ailleurs, la CPS de GBS dirige l'internalisation de la bactérie via un mécanisme d'endocytose dépendant des radeaux lipidiques et de la clathrine, alors que la CPS de *S. suis* inhibe la phagocytose via la déstabilisation des radeaux lipidiques (169, 170).

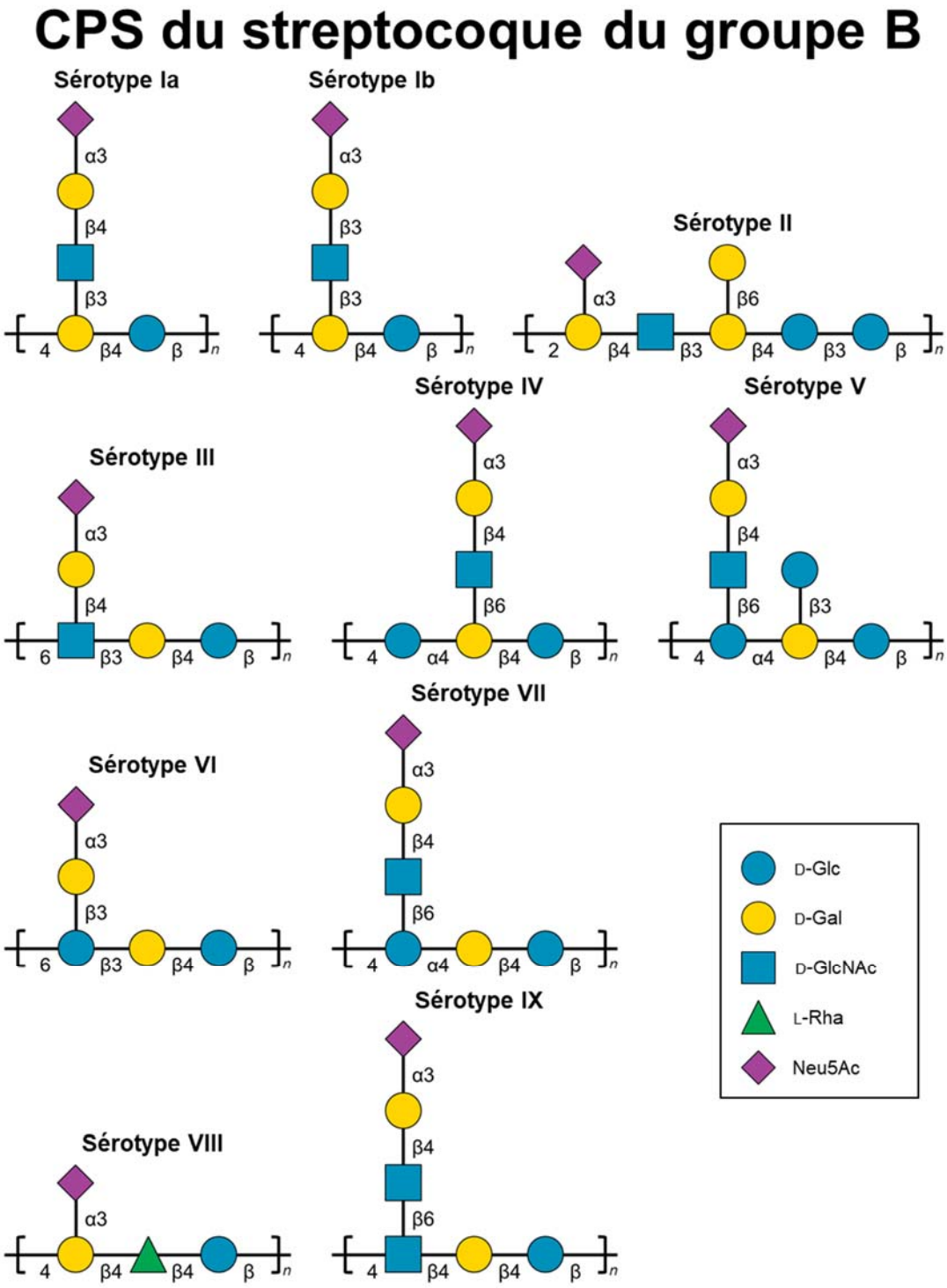


Figure 4. Diversité structurale des unités répétitives des CPS du streptocoque du groupe B

Les structures des unités répétitives pour les 10 sérotypes décrits de GBS sont présentées : types Ia & Ib (171), II (172), III (173, 174), IV (175), V (176), VI (177), VII (178), VIII (179) et IX (180). Les symboles employés pour représenter les monosaccharides suivent le système de « Symbol Nomenclature for Glycans » tel qu'établi par Varki et collaborateurs (155).

2.5. Structures et fonctions des CPS de *Streptococcus pneumoniae*

S. pneumoniae, également connu comme le pneumocoque, est une bactérie à Gram positif qui colonise normalement de manière asymptomatique le nasopharynx et les voies respiratoires supérieures des humains (181-183). C'est une cause majeure de morbidité et de mortalité du point de vue mondial, les personnes les plus à risques étant les jeunes enfants (moins de 5 ans), les personnes âgées et les patients immunocompromis, en raison de leur faible immunocompétence (184). Les maladies causées par *S. pneumoniae* forment un éventail allant d'infections mucosales, comme otites et sinusites, jusqu'à des maladies invasives plus sévères telles que pneumonies, septicémies et méningites.

Le pneumocoque est également une bactérie encapsulée pour laquelle au moins 90 sérotypes (également basés sur la structure des CPS) ont été décrits jusqu'à présent (185). Les sérotypes les plus importants de *S. pneumoniae*, soit ceux étant ciblés par les vaccins actuellement disponibles, sont présentés au **Tableau II**, et les structures des CPS pour ces sérotypes sont présentées à la **Figure 5**.

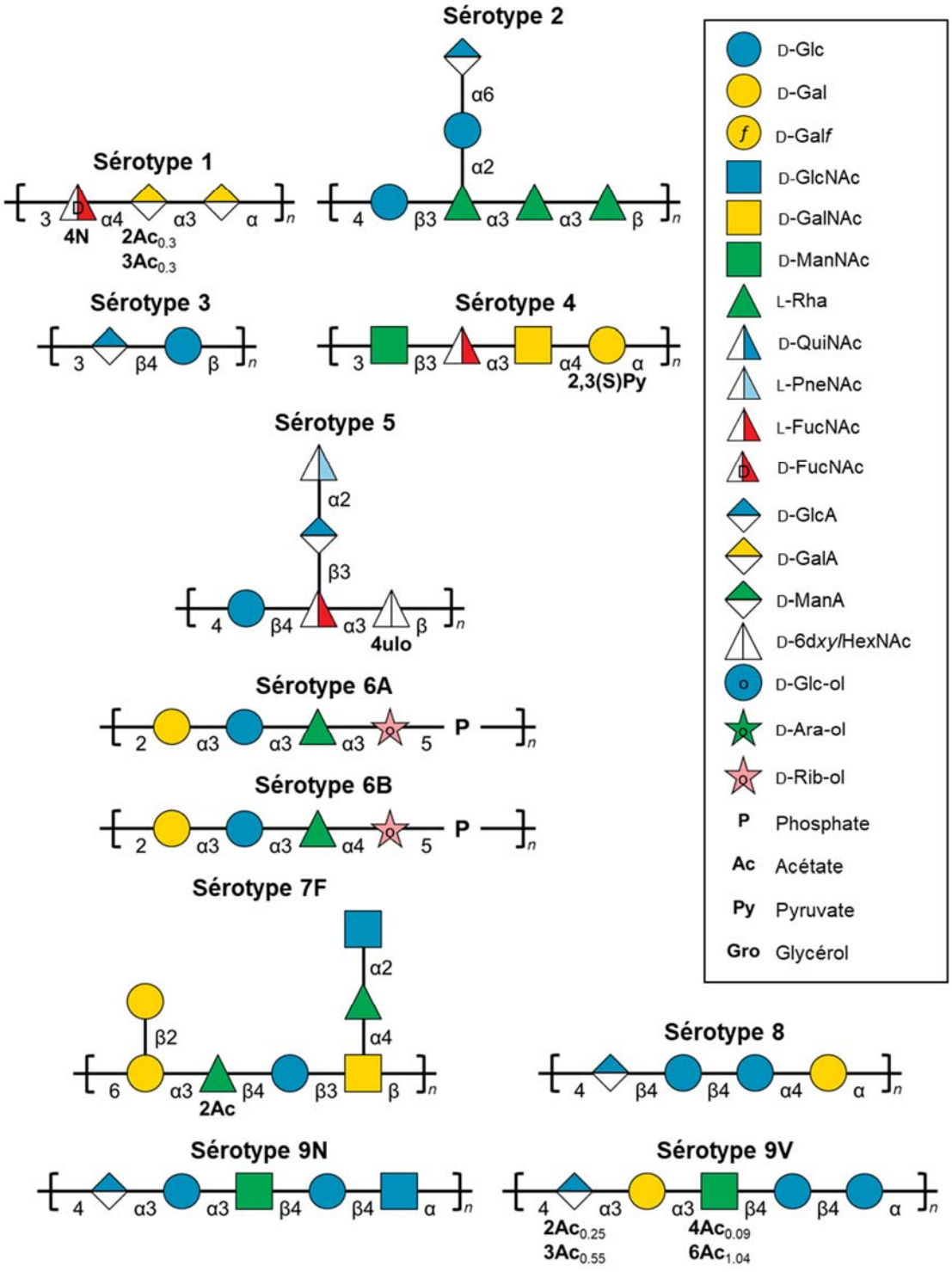
Tableau II. Contenu en sérotypes des différents vaccins commerciaux contre *S. pneumoniae*.

Tableau préparé à partir de (185, 186).

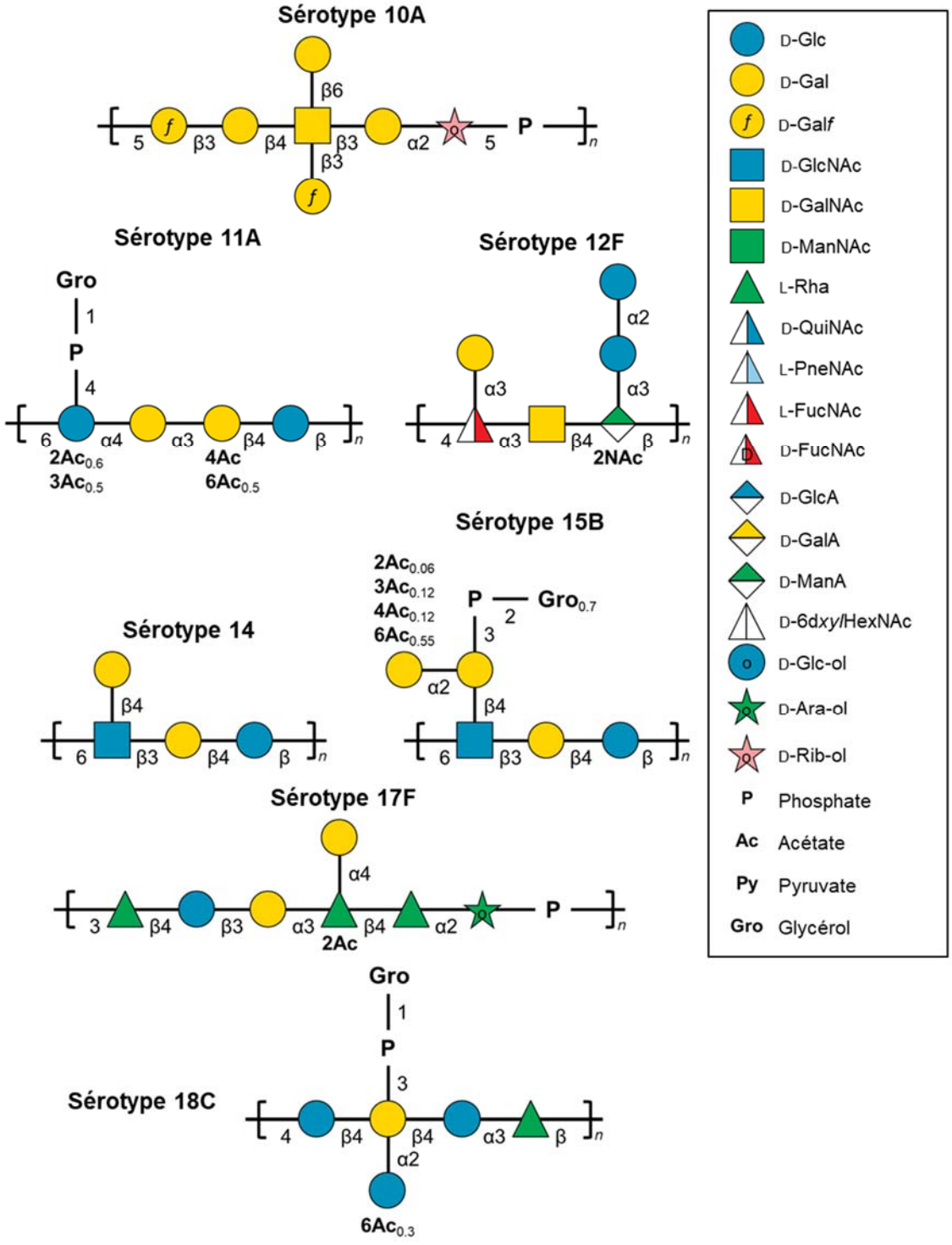
PPSV23 Pneumo 23® (1983)	**PCV7** Prevnar® (2000)	**PCV10** Synflorix® (2008)	**PCV13** Prevnar 13® (2009)
1		1	1
2			
3			3
4	4	4	4
5		5	5
			6A
6B	6B	6B	6B
7F		7F	7F
8			
9N			
9V	9V	9V	9V
10A			
11A			
12F			
14	14	14	14
15B			
17F			
18C	18C	18C	18C
19A			19A
19F	19F	19F	19F
20			
22F			
23F	23F	23F	23F
33F			

Abréviations : PPSV23, « pneumococcal polysaccharide vaccine » 23-valent; PCV7/10/13, « pneumococcal conjugate vaccine » 7/10/13-valent.

CPS de *Streptococcus pneumoniae* (1)



CPS de *Streptococcus pneumoniae* (2)



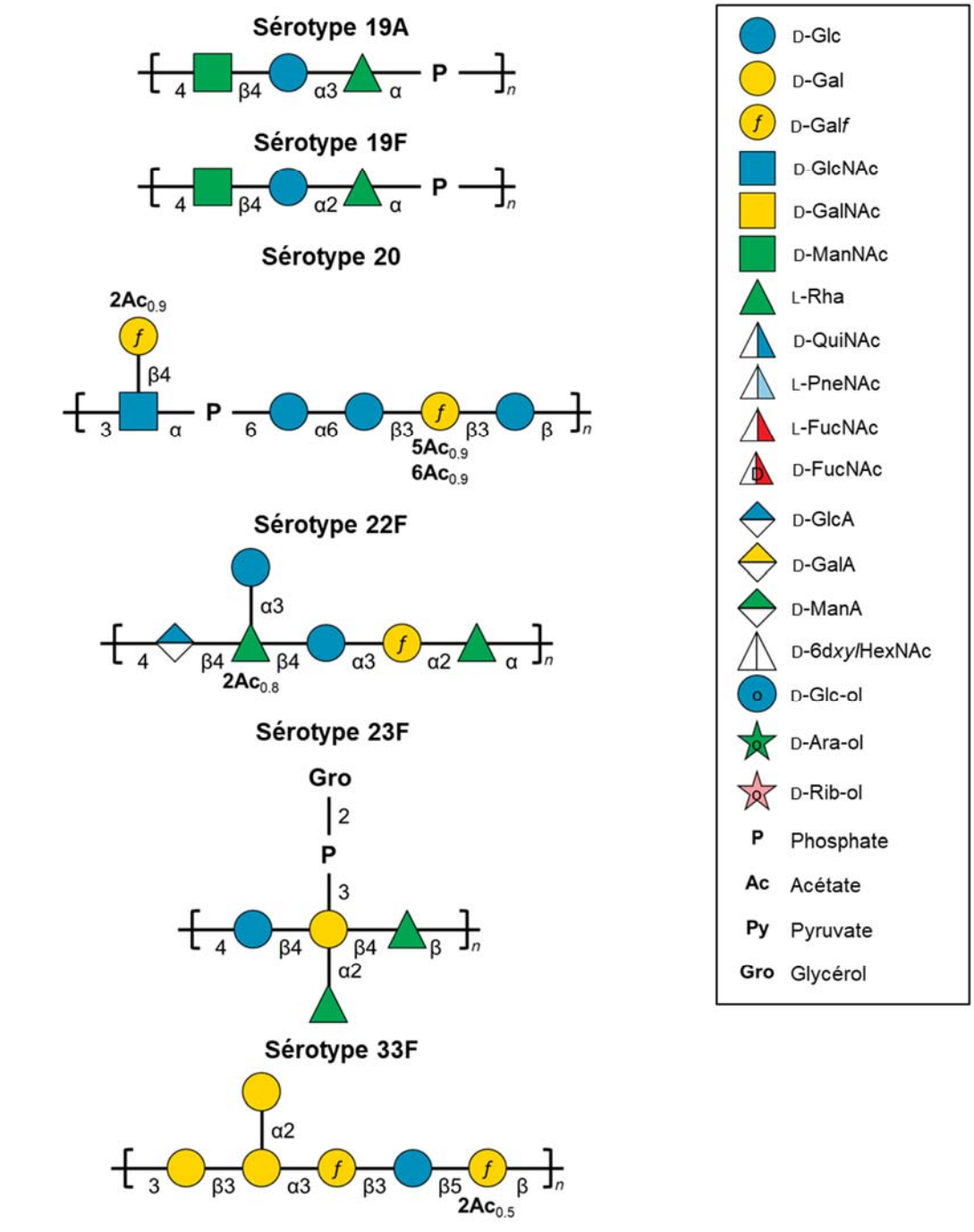


Figure 5. Diversité structurale des unités répétitives des CPS des sérotypes les plus importants de *S. pneumoniae*.

Ici sont présentés les sérotypes importants du pneumocoque, en se fiant aux sérotypes ciblés par les vaccins actuels (**Tableau II**). Il est possible de constater la grande diversité structurale de par leur composition et leur substituants (pyruvate, phosphate, glycérol et acétate) Les structures ont été recensées par Geno et al. (185). Les symboles employés pour représenter les monosaccharides suivent le système de « Symbol Nomenclature for Glycans » tel qu'établi par Varki et collaborateurs (155).
Abréviations: D-6d*xyl*HexNAc, 2-acétamido-2,6-didésoxy-β-D-*xylo*-hexose; PneNAc, N-acétylpneumosamine.

Tel que mentionné précédemment, et à l'instar de *S. suis* et de GBS, les CPS de *S. pneumoniae* sont toutes synthétisées par la voie polymérase (Wzy), exception faite des sérotypes 3 et 37 qui eux empruntent la voie de la synthase (185, 187). De plus, les structures connues révèlent que la majorité des CPS de *S. pneumoniae* possèdent une charge globale négative, ce qui a été suggéré comme facilitant la colonisation en minimisant l'élimination de celui-ci par le mucus et en repoussant les phagocytes par interactions électrostatiques (185, 188). Malgré cela, les sérotypes 7A, 7F, 14, 33F, 33A et 37 ne sont pas chargés. De plus, la CPS du sérotype 1 de *S. pneumoniae* est considéré comme étant un polysaccharide zwitterionique, compte tenu de ses deux charges positives et d'une charge négative. Alors que la vaste majorité des polysaccharides sont considérés comme des antigènes TI-indépendant, il a été démontré que les polysaccharides zwitterioniques se comportent comme des antigènes TD-dépendant, ce qui les rend d'autant plus immunogènes (189). De manière similaire à *S. suis*, la fonction primaire de la CPS de *S. pneumoniae* est de masquer la surface cellulaire pour protéger de la réponse immunitaire de l'hôte, notamment des anticorps et de l'activation du complément, ce qui est particulièrement vrai pour les sérotypes les plus invasifs/virulents (190). Encore là, les interactions entre une CPS donnée et le système immunitaire de l'hôte dépendent de la structure de celle-ci (185).

3. Mécanismes de la réponse humorale dirigée contre la CPS

Le système immunitaire permet de défendre l'organisme contre des pathogènes divers. Il est composé de nombreuses cellules et de molécules composant un réseau dynamique, capable de reconnaître et d'éliminer spécifiquement un grand nombre d'organismes étrangers (191). Il existe deux types de l'immunité : l'immunité innée et l'immunité adaptative (191). L'immunité innée fait appel à différentes cellules telles que les macrophages, les neutrophiles, les cellules NK et les DCs parmi d'autres acteurs cellulaires constituant la première ligne de défense de l'organisme contre les pathogènes. Très efficace, elle empêche dès le début la plupart des infections en éliminant la bactérie dans les quelques heures suivant son entrée dans l'organisme. Cependant, cette immunité n'est pas spécialisée dans la reconnaissance des différences subtiles entre les molécules étrangères, contrairement à l'immunité adaptative. Cette deuxième ligne de défense permet d'éliminer un pathogène qui aurait échappé à l'immunité innée ou qui persiste malgré l'activation de cette dernière. L'immunité adaptative est dépendante de l'immunité innée et prend quelques jours à se developper après l'infection initiale. Une des caractéristiques importantes de l'immunité adaptative est qu'elle est capable d'établir une réponse mémoire contre un pathogène précis, c'est-à-dire que lors d'une deuxième infection par un même pathogène, il y aura une reconnaissance et élimination très rapide de la bactérie grâce aux cellules mémoires (191).

Comme il a été précédemment mentionné que *S. suis* est hautement résistant à l'immunité innée, soit à l'action des phagocytes et du complément, notamment grâce à sa capsule, nous allons ici surtout nous intéresser au développement de la réponse adaptative jusqu'à l'obtention d'une réponse adaptative humorale opsonisante. Cette réponse opsonisante permettra à l'hôte de pouvoir combattre efficacement les infections causées par des bactéries extracellulaires encapsulées comme *S. suis*.

3.1. La cellule dendritique (DC)

Les cellules dendritiques (DCs) sont formées à partir des cellules souches de la moelle osseuse et font donc partie de la lignée myéloïde (à l'exception des DCs plasmocytoïdes). Elles peuvent être résidentes et localisées dans les organes lymphoïdes primaires et secondaires, ou migratoires et localisées dans les tissus périphériques comme la peau ou les muqueuses. Positionnées de façon stratégique dans l'organisme, les DCs s'affirment comme des cellules sentinelles et capturent l'antigène grâce à de nombreux récepteurs aux pathogènes (les PRRs pour « pattern recognition receptors »). Ces cellules sont aussi connues pour être de puissantes cellules présentatrices d'antigènes (CPA). Le terme CPA professionnelle regroupe les cellules qui expriment les molécules du Complexe majeur d'histocompatibilité de classes I et II (CMH-I et II), telles que les macrophages, les DCs et les cellules B, ce qui leur permet de présenter les antigènes aux lymphocytes T. Les DCs expriment constitutivement les molécules du CMH-I et du CMH-II et possèdent une capacité supérieure à moduler l'activation des cellules T, comparé aux autres CPAs. Ainsi, les DCs assurent le pont entre l'immunité innée et adaptative. Une fois activées, les DCs sont aussi impliquées dans le développement de la réponse humorale par la sécrétion de facteurs stimulants (tels que les cytokines) qui jouent un rôle dans la prolifération, la différenciation et la commutation isotypique des lymphocytes B. Les DCs sont une population hétérogène dont la diversité fonctionnelle des sous-populations de DCs est liée à leur état de différenciation, leur localisation, ainsi que leurs différentes interactions avec les antigènes et les cellules du système immunitaire, mais possèdent toutes le même dénominateur commun : la présentation de l'antigène (191).

3.2. La reconnaissance des pathogènes par les PRRs

Les cellules faisant partie de l'immunité innée sont capables de discriminer le soi (l'hôte) du non-soi (le pathogène) grâce à des détecteurs moléculaires qui reconnaissent des motifs structuraux variés très conservés au sein d'une espèce microbienne et généralement absents de l'hôte. Ces récepteurs, appelés récepteurs de reconnaissance de motifs moléculaires (PRRs pour « pattern recognition receptors »), sont exprimés par les cellules de l'immunité innée et reconnaissent des motifs moléculaires particuliers retrouvés chez le pathogène,

appelés motifs moléculaires associés aux pathogènes (PAMPs pour « pathogen-associated molecular patterns »).

3.2.1. Les « Toll-like receptors » (TLRs)

Les TLRs sont la famille de PRRs la plus étudiée à ce jour (192, 193). Structurellement, ce sont des protéines transmembranaires de type I caractérisées par trois types de domaines différents : (1) un domaine extracellulaire contenant des motifs de répétitions riches en leucines, qui est responsable de la liaison au ligand, (2) un domaine transmembranaire et (3) un domaine intracellulaire d'homologie au récepteur cytoplasmique de signalisation Toll/IL-1 (TIR) requis pour la transduction du signal en aval (192). Une fois le PAMP lié au TLR, le récepteur s'oligomérise afin de déclencher une cascade de signalisation conduisant à la production de médiateurs inflammatoires, dont des cytokines et des chimiokines (194).

À ce jour, dix et douze TLRs fonctionnels ont été identifiés chez l'humain et la souris, respectivement (195). Parmi eux, seuls les TLR1 à TLR9 sont conservés chez les deux espèces. Le TLR10 murin n'est pas fonctionnel dû à l'insertion d'un rétrovirus, tandis que les TLR11, TLR12 et TLR13 ont été perdus dans le génome humain (196). Les TLRs sont principalement divisés en deux sous-groupes selon leur localisation cellulaire. Les TLR1, TLR2, TLR4, TLR5, TLR6 et TLR10 sont exprimés à la surface des cellules et reconnaissent essentiellement des composants de la membrane microbienne, tels que les lipides, les lipoprotéines et les protéines (195, 196). De plus, l'activation de ces TLRs induit principalement la production de cytokines et chimiokines inflammatoires. D'autre part, les TLR3, TLR7, TLR8, TLR9 TLR11, TLR12 et TLR13 se retrouvent au niveau des membranes des endosomes (196).

Le **Tableau III** ci-dessous résume les principales molécules identifiées comme ligand respectif de chaque TLR. Ces derniers sont exprimés non seulement par des cellules immunitaires, mais aussi par d'autres types cellulaires, comme les cellules endothéliales, les

cellules épithéliales et les fibroblastes (197). Chaque type cellulaire possède son propre profil d'expression de TLRs. Ainsi, le fait de disposer de plusieurs TLRs permet à l'hôte de stimuler plusieurs types cellulaires de sorte à augmenter l'intensité de la réponse inflammatoire et à éviter une invasion du système immunitaire par un agent infectieux (196).

Tableau III. Liste des « Toll-like receptors » chez l'humain et chez la souris et leurs ligands (193, 198).

« Toll-Like Receptor »	Localisation	Ligands
TLR1	Membrane plasmique	Lipoprotéines triacylées
TLR2	Membrane plasmique	Acides lipotéichoïques Lipoprotéines
TLR3	Membrane endolysosomale	Poly(I:C) ARN double brin
TLR4	Membrane plasmique	Lipopolysaccharide
TLR5	Membrane plasmique	Flagelline
TLR6	Membrane plasmique	Lipoprotéines diacylées
TLR7/TLR8	Membrane endolysosomale	ARN simple brin
TLR9	Membrane endolysosomale	ADN bactérien (motif CpG)
TLR10	Membrane plasmique	Inconnu
TLR11 (souris)	Membrane endolysosomale	Composantes des bactéries uropathogènes
TLR12 (souris)	Membrane endolysosomale	Inconnu
TLR13 (souris)	Membrane endolysosomale	Inconnu

3.2.1.1. Signalisation par les TLRs

Suite à la reconnaissance de PAMPs, les TLRs activent différentes voies de signalisation intracellulaire permettant d'induire une réponse inflammatoire (195). Ces voies de signalisation débutent par le recrutement de différentes protéines adaptatrices présentes à l'intérieur de la cellule, dont les plus importantes sont le « Myeloid differentiation primary response 88 » (MyD88) et le « TIR-domain-containing adapter-inducing IFN-β » (TRIF) (192, 195). Leur recrutement mène donc à l'initiation de deux voies principales de signalisation, soit la voie MyD88-dépendante et la voie TRIF-dépendante (194). Bien que les cascades de signalisation intracellulaires varient entre ces deux voies, elles mènent toutes deux à la production de médiateurs pro-inflammatoires (193). En effet, la voie MyD88-dépendante entraîne principalement la production de cytokines et chimiokines pro-inflammatoires, tandis que la voie TRIF-dépendante induit la production des IFNs de type I (193).

3.2.1.2. La signalisation MyD88-dépendante

À l'exception du TLR3, tous les TLRs nécessitent l'implication de la protéine MyD88 pour initier leur signalisation (193). Cette voie débute par l'association de la protéine adaptatrice MyD88 au domaine cytoplasmique TIR des TLRs activés (193). Cependant, les TLR1/2, TLR2/6 et TLR4 ont également besoin de la protéine adaptatrice TIRAP, qui agit comme pont de liaison entre le domaine TIR de ces TLRs et la protéine MyD88 (192). Une fois la liaison de MyD88 achevée, cette protéine s'associe avec « IL-1 receptor-asssociated kinase » (IRAK) 4 via l'interaction des « death domains » retrouvés chez les deux molécules (195, 199). Le complexe MyD88/IRAK4 stimule l'autophosphorylation d'IRAK4 et le recrutement de deux autres membres de la famille, soit IRAK1 et IRAK2. La forte proximité entre les trois molécules IRAKs permet à IRAK4 d'activer IRAK1 et IRAK2 en les utilisant directement comme substrat pour son activité kinase (192). L'activation des molécules IRAKs permet le recrutement de « TNF receptor-associated factor » (TRAF) 6, ainsi que de l'ubiquitine ligase E3 (196). Ces derniers forment alors un complexe qui, une fois activé, est libéré dans le cytoplasme, entraînant ainsi la libération du facteur nucléaire-κB (NF-κB), qui se transloquera au noyau (192). De plus, cette voie mène aussi à la phosphorylation des protéines kinases activées par les mitogènes (MAPK) (192). Leur activation aura pour

conséquence le déclenchement de la réponse inflammatoire en favorisant la transcription des gènes codant pour les cytokines et chimiokines pro-inflammatoires (196, 199).

3.3. La synapse biologique entre la DC et les lymphocytes T *helper*

3.3.1. La maturation des DCs

Après avoir capturé l'antigène par phagocytose, endocytose ou pinocytose, les DCs passent d'un phénotype de capture d'antigènes à celui de présentatrices d'antigènes aux cellules T. Au cours de cette maturation, certaines caractéristiques sont perdues, et d'autres apparaissent. Par exemple, les capacités de phagocytose ou pinocytose sont diminuées, tandis que l'expression de CMH et des molécules de costimulation, nécessaires à la présentation de l'antigène aux lymphocytes T, augmentent significativement. De plus, des molécules d'adhésion ainsi que des récepteurs pour certaines chimiokines émergent à la surface des DCs, qui leurs permettent de migrer du site d'infection vers les organes lymphoïdes secondaires pour aller à la rencontre des lymphocytes T (191).

Il existe deux classes de CMH. Le CMH de classe I (CMH-I) est exprimé par toutes les cellules nucléées de l'organisme, ainsi que les DCs. Il permet de présenter les antigènes intracellulaires (plus précisément les antigènes d'une cellule infectée par un pathogène intracellulaire) aux lymphocytes T CD8+ cytotoxiques, impliqués dans des réponses immunitaires antivirales principalement. Quant au CMH de classe II (CMH-II), il est exprimé seulement sur les CPAs professionnelles, et présente des antigènes exogènes provenant, entre autres, de bactéries extracellulaires comme *S. suis* aux lymphocytes T CD4+ (ou *helper* [Th]). De plus, la surexpression du CMH est de pair avec celle des molécules de costimulation CD80/86 et CD40 (191). Compte tenu du sujet de cette thèse, nous allons nous concentrer plus particulièrement à la présentation des antigènes via le CMH-II aux lymphocytes Th.

3.3.2. La présentation de l'antigène

Une fois dans les organes lymphoïdes secondaires, la DC présente l'antigène aux lymphocytes T *helper* (Th). Ceci se fait grâce au CMH-II et par la surexpression de molécules de costimulation telles que CD40 et CD80/66. La présentation de l'antigène pour activer les lymphocytes T s'effectue à l'aide de trois signaux. Le premier signal correspond au complexe CMH/antigène exprimé à la surface de la CPA qui est reconnu par le récepteur des cellules T (TCR) spécifique à l'antigène. Le deuxième signal correspond à l'engagement des molécules de costimulation : CD40 exprimé par les DCs se liant au CD40-ligand (CD40L) exprimé par les lymphocytes T, et CD80/86 exprimé par les DCs se liant au CD28 exprimé par les lymphocytes T.

Le troisième signal, soit la sécrétion de cytokines, représente une étape cruciale dans l'influence de l'activation des lymphocytes Th. En effet, ces derniers se différentieront en divers sous-types de lymphocytes Th effecteurs, dont : Th1 et Th2 (décrits dans la section suivante), en fonction de l'antigène présenté et des cytokines produites par les DCs et autres cellules de l'immunité innée (**Tableau IV**). Les cytokines sécrétées peuvent être pro-inflammatoires (telles que IL-1β et TNF-α), anti-inflammatoires (telle que l'IL-10), ou encore des chimiokines. En plus de leur rôle dans le processus d'inflammation, ces cytokines influencent grandement l'activation des lymphocytes T et B, en jouant un rôle crucial dans le devenir de la réponse humorale.

Tableau IV. Principales cytokines jouant un rôle dans le développement de la réponse immunitaire de type 1 et de type 2.

Cytokine	Produite par	Type d'immunité
IL-12 TNF IL-1β	Cellules dendritiques et autres cellules de l'immunité innée	Type 1
IFN-γ TNF IL-2	Lymphocytes Th1	
IL-6 IL-10	Cellules dendritiques et Lymphocytes Th2	Type 2
IL-4	Lymphocytes Th2	

Abréviations : IL, Interleukine; TNF, « Tumor-necrosis factor »; IFN, Interféron; Th1/2, « T helper 1/2 ».

À titre d'exemple, l'IL-12 est universellement décrite comme étant un médiateur clé dans le déclenchement de la réponse immunitaire de type 1. Il s'agit d'un hétérodimère comprenant une sous-unité p35 (de 30–33 kDa) et une autre p40 (de 35–44 kDa). L'hétérodimère p70 est la forme active de la cytokine (200). Pendant le processus de la présentation de l'antigène aux lymphocytes Th, l'IL-12, sécrétée par les DCs, se lie à un récepteur présent à la surface de ces lymphocytes et initie leur différentiation en lymphocytes CD4+ Th1. De ce fait, l'IL-12 inhibe le développement de la réponse immunitaire de type 2 en agissant comme antagoniste à la cytokine IL-4 (cytokine Th2) (201). De plus, le TNF constitue un cofacteur pour l'IL-12 afin de promouvoir le développement adéquat d'une réponse immunitaire de type 1 (202). Au contraire, l'IL-6 joue un rôle dans le développement de la réponse immunitaire de type 2. Sécrétée par les DCs, elle se lie ensuite sur un récepteur présent à la surface des lymphocytes Th naïfs et permet la sécrétion autocrine de l'IL-4 par ces derniers, facilitant ainsi leur différentiation en lymphocytes CD4+ Th2 (203).

3.4. L'activation et la différenciation des lymphocytes Th

Les cellules Th reconnaissent un antigène présenté par le CMH-II (l'antigène correspond à une bactérie extracellulaire comme *S. suis*, à titre d'exemple). Les lymphocytes T ne peuvent reconnaître un antigène seulement lorsque celui-ci est présenté via le CMH par une CPA. Par la suite, ces cellules T prolifèrent et se différencient en divers types de cellules T effectrices et mémoires (191). Comme il a été mentionné précédemment, pour les lymphocytes Th, plusieurs sous-types de cellules Th effectrices existent, dont les Th1 et les Th2, qui influencent le développement d'une réponse humorale de type 1 et de type 2, respectivement (191).

3.4.1. Les lymphocytes Th1

Comme déjà mentionné dans les sections précédentes, le développement des cellules Th1 débute avec la production d'IL-12 par les DCs. L'IL-12 agit directement sur les cellules T pour induire leur différentiation en cellules Th1. La sécrétion d'IFN-γ (principale cytokine de type 1) par les cellules Th1 activées agit comme une boucle de rétroaction positive en stimulant la production de cytokines par les DCs. L'IFN-γ possède l'habilité à inhiber la voie de différenciation des cellules Th2. Les cellules Th1 stimulent la phagocytose et la lyse des pathogènes par les phagocytes; en effet, l'IFN-γ est un puissant activateur des macrophages. L'étape critique de la réponse Th1 demeure l'activation des cellules B par l'IFN-γ et l'IL-2 pour mener à la production d'anticorps IgG de type 1 protecteurs. Ces anticorps favorisent l'opsonophagocytose des pathogènes par l'engagement des récepteurs Fc des phagocytes et par l'activation du complément. Pour ces raisons, les cellules Th1 et l'immunité de type 1 sont souhaitées pour l'élimination de pathogènes extracellulaires comme *S. suis* (191).

3.4.2. Les lymphocytes Th2

Les mécanismes impliqués dans la polarisation de la réponse Th2 sont moins bien connus que ceux de la réponse Th1. L'IL-4 est la principale cytokine Th2 sécrétée de façon autocrine par les lymphocytes Th et initie leur différenciation en cellules Th2 effectrices, mais les cellules impliquées dans la production initiale de cette cytokine sont encore inconnues

(191). Il a cependant été décrit que l'IL-6 produit par les CPAs joue un rôle dans le développement de la réponse Th2 (203). La production d'IL-10 par les CPAs pourrait aussi induire indirectement la différenciation des cellules Th2, en inhibant la synthèse d'IL-12 et donc le développement d'une réponse de type 1 (204). Une fois différenciés en lymphocytes Th2, d'autres cytokines sont sécrétées telles que l'IL-5, l'IL-6, l'IL-13 et l'IL-4, et influencent l'activation des cellules B ainsi que la commutation isotypique des anticorps de type 2. Les anticorps produits sont plus spécialisés pour jouer un rôle dans la neutralisation de toxines, l'immunité mucosale ainsi que dans les réactions allergiques (191). Ils sont donc moins protecteurs face à une bactérie extracellulaire et encapsulée comme *S. suis* (135).

3.5. L'activation des lymphocytes B

Les lymphocytes B arrivent à maturation dans la moelle osseuse, et lorsqu'ils la quittent, chacun exprime à sa surface une molécule d'anticorps membranaire (ou immunoglobine membranaire [mIg]) faisant partie du récepteur de la cellule B (ou BCR), capable de reconnaître un antigène spécifique et unique à chaque lymphocyte. Lorsqu'une cellule B naïve rencontre un antigène pour la première fois grâce à son BCR, celle commence à se diviser rapidement (expansion clonale). Les cellules qui résultent de cette division finiront par se différencier en cellules B mémoires et en plasmocytes. Les cellules B mémoires ont une longue durée de vie et continuent à exprimer le même anticorps membranaire que la cellule B naïve dont elles sont issues. Les plasmocytes produisent de nombreux anticorps sous forme sécrétés (191). L'activation des lymphocytes B peut se faire selon deux voies différentes, à savoir la voie TD et la voie TI. L'activation des cellules B par la voie TD requiert l'interaction avec les lymphocytes Th, alors que pour la voie TI cette interaction n'est pas requise.

3.5.1. L'activation T-dépendante (TD)

Suite à l'activation initiale, les lymphocytes B en prolifération, nommés blastes, vont procéder selon une de deux voies indépendantes pour leur migration et différentiation. En premier lieu, les cellules B activées vont migrer vers les régions extrafolliculaires, où leur prolifération rapide et leur différentiation en plasmablastes et en plasmocytes surviendra. Ces

« antibody-secreting cells » transitoires fourniront une première source d'anticorps spécifiques (principalement des IgM) en réponse au pathogène / antigène initial et une protection rapide. Cette réponse humorale primaire cherche à protéger l'organisme durant le développement de la réponse germinale qui prendra plusieurs jours (205). Ceci étant dit, la réponse humorale TD est fortement associée à la formation d'un centre germinatif (GC), et à la commutation isotypique, à la maturation d'affinité et au développement de cellules mémoire qui en découle et qui seront décrits ci-bas.

3.5.1.1. Formation du centre germinatif

En second lieu (même si son déroulement est simultané à la réaction extrafolliculaire), les blastes se retrouveront au sein des follicules d'un organe lymphoïde secondaire où ils vont débuter la formation d'un centre germinatif (GC). En effet, les lymphocytes B matures naïfs sont en recirculation constante au travers des organes lymphoïdes secondaires à la recherche de signes d'une infection, et lors de l'atteinte des follicules, vont circuler rapidement à l'intérieur de ceux-ci à la recherche de leur antigène. Lors de la rencontre avec son antigène, le lymphocyte B va migrer à la frontière entre le follicule de cellules B et les zones de lymphocytes T (suite à l'augmentation soudaine de l'expression du récepteur chimiokinique « C–C motif receptor 7 » (CCR7) pour aller recruter l'aide des lymphocytes Th. L'interaction T-B permettra l'induction de la formation d'un GC notamment suite aux interactions CD40 – CD40L. Typiquement, on retrouve 1 à 6 clones à coloniser ces follicules, ce qui fait que les réactions germinales sont de nature oligoclonale (205).

3.5.1.2. Commutation isotypique des immunoglobulines

En plus d'exprimer CD40L, les lymphocytes Th activés sécrètent également des cytokines qui serviront à induire la prolifération des cellules B et leur différentiation, en plus de jouer un rôle central dans le déclenchement de la commutation isotypique des immunoglobulines (IgM → IgG, IgA ou IgE). Typiquement, les voies de signalisation déclenchées par ces cytokines vont mener à la translocation de l'enzyme « activation-induced cytidine deaminase » (AID) vers une des séquences de recombinaison situées à l'extrémité 5′

de chacun des gènes codant pour les régions constantes des chaînes lourdes d'immunoglobulines γ, α et ε. Ainsi, AID va déclencher les événements de recombinaison homologue d'ADN menant à la commutation isotypique. Concrètement, au cours de ces recombinaisons, le gène codant pour la chaîne lourde μ (IgM) sera remplacé par celui d'une des chaînes lourdes γ (IgG), α (IgA) ou ε (IgE). Le processus de commutation isotypique peut être initié en quelques jours suite à l'activation initiale du lymphocyte B. De plus, bien que la commutation isotypique peut survenir au sein d'un GC, celle-ci n'est pas limitée à cette phase de la réponse humorale (205).

3.5.1.3. Maturation d'affinité

Les GCs sont le site primaire où la diversification et la maturation d'affinité des lymphocytes B activés se produisent. Alors que celles-ci prolifèrent au sein des GCs, elles acquièrent de hauts taux de mutations dans les régions variables de leurs immunoglobulines par hypermutation somatique. Tout comme pour la commutation isotypique, l'enzyme AID est impliquée dans ce processus qui résulte en l'introduction de mutations aléatoires affectant l'affinité et la spécificité du BCR. Les clones de cellules B au sein du GC qui expriment des variants avec une liaison plus forte à l'antigène sont positivement sélectionnés et prolifèrent, alors que les variants pour lesquelsqui les changements résultent en des liaisons plus faibles ne sont pas sélectionnés, entraînant leur mort par apoptose. Ceci résulte en une augmentation de l'affinité moyenne des anticorps sécrétés au fur et à mesure que la réponse progresse (205). Les clones de cellules B sélectionnés au sein des GCs vont pouvoir se différencier soit en cellules B mémoire ou en plasmocytes à longue durée de vie.

3.5.1.4. Mémoire humorale

Une des grandes caractéristiques de la réaction germinale est dans la génération d'une mémoire humorale. Les cellules B mémoire ont été démontrées comme étant capables de persister pour de très longues périodes de temps après exposition à l'antigène et pour recirculer au travers des organes lymphoïdes secondaires, en plus de coloniser la zone marginale de la rate. En raison de leur sélection dans les GCs, les cellules B mémoire

expriment des immunoglobulines portant des mutations somatiques et donc sont capables de répondre très rapidement suite à une réexposition à l'antigène avec des anticorps de haute affinité. La mémoire humorale à long terme est également maintenue par une population de plasmocytes à longue durée de vie, sélectionnés également dans les GCs, et qui sont maintenant résidentes de la moelle osseuse. Ces plasmocytes continueront de sécréter tout au long de leur vie leur immunoglobuline de haute affinité afin de protéger l'hôte (205).

3.5.2. L'activation T-indépendante (TI)

Les mécanismes de l'activation par la voie TI sont moins bien connus que la précédente. En bref, comme son nom l'indique, cette activation se manifeste indépendamment des lymphocytes T. Les antigènes TI sont habituellement des molécules de nature polysaccharidique, telle que la CPS de *S. suis*. La principale caractéristique des antigènes TI est leur incapacité à se complexer au CMH-II, et par conséquent ne peuvent être présentés aux lymphocytes Th pour obtenir leur aide, du coup les rendant beaucoup moins immunogènes que les antigènes protéiques TD (206). Une exception serait les polysaccharides zwitterioniques (porteurs de charges négatives et positives) qui eux peuvent être présentés par le CMH-II, ce qui les rend beaucoup plus immunogènes que les polysaccharides « réguliers » (189).

En l'absence des cellules Th, deux signaux sont requis pour pouvoir activer les lymphocytes B et la sécrétion d'anticorps. Le premier signal consiste en le « cross-linking » des mIg par un antigène multivalent (comme une CPS), qui permettra d'activer la cellule B CPS-spécifique. Seul, ce premier signal résulte en une faible production d'IgM, en l'absence de commutation isotypique et en l'absence de mémoire. Suite à un second signal, comme la signalisation via les TLRs ou la production de cytokines et chimiokines par une CPA préalablement activée (plus particulièrement dans le contexte d'une bactérie entière encapsulée que d'un polysaccharide soluble), les cellules B activées par un antigène TI peuvent entreprendre la commutation isotypique (résultant en la production d'IgG), sécrètent des quantités substantielles d'immunoglobulines et génèrent des cellules mémoires de faible affinité (207).

3.5.3. Potentiel des lymphocytes Tcarb et d'autres cellules immunitaires

En plus de ce qui a été présenté précédemment, d'autres populations de cellules immunitaires peuvent intervenir dans le développement de la réponse immunitaire adaptative face aux glycanes. Des travaux relativement récents de 2011 par le laboratoire du Dr. Kasper ont mis à jour l'existence de clones glycane-spécifique de cellules T, nommés Tcarb (208). La contribution de ces Tcarb ainsi que leur mécanisme d'action dans la réponse TD face aux vaccins glycoconjugués sera développé plus loin dans la section **4.1.**.

En plus des Tcarb, d'autres cellules comme les cellules NK et « natural killer T » (NKT) sont connues pour reconnaître les glycanes et pourraient contribuer indirectement à la réponse anti-CPS TI (207, 209). En effet, par la reconnaissance de PAMPs bactériens, les cellules NK sécrètent de grandes quantités de cytokines, comme l'IFN-γ, ce qui pourra contribuer à l'activation TI des lymphocytes B (210). De plus, les cellules NKT, quant à elles, peuvent reconnaître les glycolipides présentés par CD1, ce qui leur permet de s'activer et de fournir les signaux nécessaires aux lymphocytes B (209). Alors que les NKT reconnaissent les glycolipides de GBS, elles sont incapables de reconnaître les glycolipides isolés d'une souche de sérotype 1 de *S. suis* (211). Néanmoins, une seconde étude *in vivo* suggère que les NKT peuvent être activées à de très faibles niveaux lors d'une infection par le sérotype 2 de *S. suis* (212). Finalement, le rôle de ces trois types cellulaires dans les réponses anti-CPS n'a encore jamais été étudié pour *S. suis*.

3.6. La production d'anticorps

Tel que mentionné précédemment, les cellules B naïves n'expriment que l'IgM, tandis que les cellules B matures et mémoires expriment principalement les Ig de type IgG (aussi IgA et IgE). L'IgM peut se présenter sous deux formes : la forme monomérique lorsqu'il est exprimé sous forme membranaire des cellules B, ou la forme pentamérique lorsqu'il est sécrété par les plasmocytes. L'IgM est le premier isotype d'anticorps produit et est fortement opsonisant, étant capable d'activer le complément (191). L'IgA est principalement impliqué dans l'immunité mucosale, alors que l'IgE est impliqué dans les défenses face aux parasites ainsi que dans les réponses allergiques (191). L'IgG est la classe d'anticorps la plus abondante

dans le sérum et possèdent un atout d'opsonisation. Ils existent différentes sous-classes d'IgGs qui jouent des rôles distincts plus ou moins caractérisés selon les espèces (**Tableau V**).

En effet, chez la souris, les sous-classes IgG2a/2c, IgG2b et IgG3 sont caractérisées comme de type 1 (213). Leur production est donc associée au développement d'une réponse immunitaire de type 1, découlant de la différenciation des lymphocytes Th en Th1 suite à la présentation de l'antigène par les CPAs. Ces sous-classes IgG de type 1 jouent un rôle important dans l'élimination des pathogènes par opsonophagocytose, soit via l'activation du complément et/ou l'engagement des récepteurs Fc des phagocytes. Ainsi, ce type d'anticorps est déterminant pour la protection contre les bactéries extracellulaires et encapsulées, telle que *S. suis* (135).

Au contraire, l'IgG1 murin est décrit comme un anticorps associé à la réponse immunitaire de type 2. Les anticorps produits dans le cadre de réponses immunitaires Th2 sont plus spécialisés pour jouer un rôle dans la neutralisation de toxines, l'immunité mucosale ainsi que dans les réactions allergiques (191). Ils sont donc moins protecteurs face à une bactérie extracellulaire et encapsulée comme *S. suis* (135).

À titre comparatif, chez l'homme, les sous-classes d'IgG de type 1 sont les IgG1 et IgG3 alors que les sous-classes de type 2 sont les IgG2 et IgG4.

Cependant, contrairement à ce qui est rapporté chez la souris et chez l'homme, le concept des sous-classes IgG de type 1/type 2 chez le porc demeure largement inconnu, notamment en raison du manque d'outils immunologiques appropriés. Auparavant, Crawley et al. avait rapporté que les cytokines Th1 IFN-γ et IL-12 induisaient une réponse anticorps principalement IgG2 (214), et que l'IgG2 était plus efficace pour activer le complément que l'IgG1; ainsi, les IgG2 porcins seraient réputés être plus opsonisants que les IgG1 (215). Cependant, basé sur des analyses bio-informatiques, on suspecte aujourd'hui l'existence des sous-classes putatives d'IgG porcins suivantes et de leurs variants alléliques : IgG1a/b, IgG2a/b, IgG3, IgG4a/b, IgG5a/b and IgG6a/b (216). De plus, il a été rapporté en 2010 que les anticorps monoclonaux anti-IgG1 et anti-IgG2 porcins, les seuls outils disponibles

actuellement, reconnaîtraient plutôt des groupes de sous-classes au lieu de sous-classes spécifiques (217). Ceci permettrait d'expliquer la confusion parmi les études tentant d'établir une corrélation entre une sous-classe d'IgG et protection chez le porc. Il a également été suggéré que l'induction de l'IgG3 porcin serait important pour protéger contre *S. suis*, puisque des analyses bio-informatiques ont rapporté que cette sous-classe serait la plus susceptible d'activer le complément et de se lier aux récepteurs Fcγ (FcγR), les deux composantes majeures de l'opsonophagocytose (216). Il serait donc nécessaire de développer de meilleurs outils immunologiques afin d'élucider le rôle protecteur des différentes sous-classes d'IgG porcins.

Tableau V. Les différents isotypes et sous-classes d'Ig ainsi que leurs rôles dans la protection contre les bactéries extracellulaires encapsulées selon les espèces (213, 216, 218-220).

SOURIS					
Anticorps	**IgM**	**IgG1**	**IgG2a/2c**	**IgG2b**	**IgG3**
Type d'immunité	—	Type 2	Type 1		
Activation du complément	+++	+/−	+++	+++	+++
Liaison aux récepteurs Fcγ activateurs	−	−	++	++	+++
Liaison au récepteur inhibiteur FcγRIIB	−	++	+	++	−
Affinité relative à l'antigène	+	+++	++	++	++
PORC					
Anticorps	**IgM**	**IgG1**	**IgG2**	**IgG3**	**IgG4/5/6**
Type d'immunité	—	Type 2 ?	Type 1 ?	?	?
Activation du complément	+++	+	++	++ (?)	?
Liaison aux récepteurs Fcγ activateurs	−	+/−	+	++ (?)	?
HUMAIN					
Anticorps	**IgM**	**IgG1**	**IgG3**	**IgG2**	**IgG4**
Type d'immunité	—	Type 1		Type 2	
Activation du complément	+++	+++	+++	+	−
Liaison aux récepteurs Fcγ activateurs	−	+++	+++	+/−	+/−

3.7. Mécanismes effecteurs de la réponse humorale contre les bactéries extracellulaires

L'immunité humorale est médiée par les anticorps sécrétés et sert principalement de défense contre les microbes extracellulaires et les toxines microbiennes. L'immunité humorale est une forme de protection qui peut être transférée d'individus immunisés à des individus naïfs via le sérum. Les vaccins utilisés présentement induisent la protection principalement en stimulant la production d'anticorps. Les principales fonctions effectrices d'une réponse humorale sont la neutralisation de microbes et de toxines (ne sera pas développée dans le cadre de cette thèse, car mécanisme non pertinent à *S. suis*) et, plus particulièrement, l'opsonophagocytose via l'activation du complément et via la phagocytose médiée par les FcγR (191).

L'opsonisation se définit par l'action de « marquer » ou de recouvrir une particule afin de promouvoir sa phagocytose, alors que les opsonines (anticorps et/ou protéines du complément) sont les substances qui performent l'opsonisation (191).

3.7.1. Opsonisation médiée par les anticorps et phagocytose

En l'absence d'anticorps spécifiques, *S. suis* est hautement résistant à l'activité bactéricide/phagocytaire des phagocytes mononucléés (macrophages, monocytes et DCs) et des neutrophiles. Cependant, l'opsonisation par les anticorps, et plus particulièrement par les IgG, permettra d'engager les récepteurs Fc spécifiques pour les chaînes lourdes des IgG (nommés FcγR). Ces récepteurs sont exprimés par différentes populations de leucocytes : ceux qui sont principalement impliqués dans la phagocytose sont exprimés majoritairement par les macrophages et les neutrophiles. Tous les FcγR possèdent une chaîne α qui permet la liaison du ligand à son récepteur; les différences structurales pour cette chaîne α expliquent les différences de spécificité et d'affinité de chaque FcγR pour chaque sous-classe d'IgG. Ces récepteurs sont optimalement activés par des anticorps liés à leur antigène, et non pas par les anticorps libres en circulation. De plus, à l'exception de FcγRII, les chaînes α sont associées à des chaînes polypeptidiques permettant la transduction du signal (191, 218).

FcγRI est le FcγR majeur des phagocytes. Exprimés par les macrophages et les neutrophiles, celui-ci lie avec haute affinité les sous-classes d'IgG de type 1. Sa chaîne α est associée à une protéine de signalisation homodimèrique nommée chaîne γ des FcR. Cette chaîne γ possède une courte séquence N-terminale extracellulaire et une longue séquence C-terminale intracellulaire, incluant notamment un motif activateur homologue au « immunoreceptor tyrosine-based activation motif » (ITAM) qui permet de coupler l'aggrégation et le « cross-linking » des récepteurs (suite à l'engagement d'antigènes multivalents comme une bactérie opsonisée) à l'activation de protéines tyrosine kinases qui déclencheront les cascades de signalisation intracellulaires (191). Ces cascades de signalisation chez les leukocytes entraîneront la production de cytokines, de médiateurs inflammatoires et de produits microbicides (espèces réactives d'oxygène, oxyde nitrique et enzymes hydrolytiques), la mobilisation du cytosquelette menant à la phagocytose, l'exocytose des granules et la migration des cellules vers le site de l'infection (191, 218).

D'autres FcγR, commes les FcγRIIA, FcγRIIC, FcγRIIIA et FcγRIIIB humains, et les récepteur murins FcγRIII et FcγRIV, peuvent lier les différentes sous-classes d'IgG avec une affinité plus faible comparativement à FcγRI et permettent également d'activer les phagocytes grâce à leurs ITAM (218). De plus, autant chez l'humain que chez la souris, le FcγRIIB est un récepteur inhibiteur (motif intracellulaire « immunoreceptor tyrosine-based inhibition motif » [ITIM]) exprimé par les cellules myéloïdes (DCs, neutrophiles, macrophages et mastocytes) et les cellules B. Les sous-classes d'IgG murins ayant la meilleure affinité pour ce récepteur sont les IgG de type 2. Comme le FcγRIIB joue un rôle immunorégulateur, celui-ci ne sera pas discuté davantage dans le cadre de cette thèse (191, 218).

3.7.2. Activation du complément

En l'absence d'anticorps spécifiques, *S. suis* est hautement résistant au système du complément, notamment grâce à deux protéines permettant le recrutement du facteur H à sa surface, une protéine soluble inhibitrice du complément (221). Cependant, l'opsonisation par les anticorps, et plus particulièrement par les IgM et les sous-classes d'IgG de type 1, permet

également l'activation du complément par la voie classique. L'activation du complément par la voie alternative et la voie des lectines ne sera pas discutée davantage dans le cadre de cette thèse, puisque ces deux voies ne sont pas pertinentes à *S. suis*.

La voie classique est initiée par la liaison de la protéine du complément C1 aux régions Fc des IgM et IgGs qui sont liés à leur antigène. Tel que déjà mentionné, les sous-classes d'IgG de type 1 sont bien plus efficaces que les sous-classes de type 2 dans l'activation du complément. C1 est un large complexe protéique multimérique composé des sous-unités C1q, C1r et C1s : C1q lie l'anticorps, et C1r et C1s sont des protéases. La sous-unité C1q forme un arrangement radial de six chaînes (semblable à un parapluie), chacune possédant une tête globulaire. Cet hexamère est responsable de la reconnaissance moléculaire et lie spécifiquement les chaînes des régions Fc μ et certaines γ. Comme précédemment avec les récepteurs Fc, seulement les anticorps liés à leur antigène permettent l'activation de la voie classique du complément, la raison étant que chaque C1q doit lier au moins deux régions Fc. Dans le cas de l'IgG pour lequel qui la région Fc ne possède qu'un seul site de liaison à C1q, il faut qu'un minimum de deux régions Fc soit accessibles à C1q pour permettre l'activation du complément; ceci se produit seulement lorsque les anticorps lient un antigène multivalent, comme une CPS. Dans le cas de l'IgM libre en circulation, bien que celui-ci soit pentamérique (donc multiples régions Fc pour un seul anticorps), il se trouve dans une configuration (forme planaire) qui prévient la liaison à C1q. La liaison de l'IgM à son antigène induit un changement de conformation (forme d'agrafe) qui permet d'exposer les sites de liaison à C1q et permet ainsi la liaison. De plus, comme l'IgM est pentamérique, un seul IgM peut lier en même temps deux molécules de C1q, et c'est une des raisons pour lesquelles l'IgM est plus efficace que les IgGs dans l'activation du complément (191, 222).

C1r et C1s sont des sérines protéases qui forment un tétramère comprenant deux molécules de chaque sorte. La liaison d'un minimum de deux têtes globulaires de C1q aux régions Fc des anticorps mène à l'activation enzymatique de C1r, qui clive et active C1s. Activée, C1s clive la prochaine protéine du complément dans la cascade, soit C4, pour générer C4b. C4 étant homologue à C3, celle-ci va former un lien covalent avec soit le complexe antigène-anticorps ou avec la surface cellulaire adjacente à l'anticorps lié. Cet attachement de

C4b permet de s'assurer que l'activation du complément se déroule au site de liaison des anticorps. La prochaine protéine du complément C2 forme alors un complexe avec C4b ancré à la surface et est clivé par une C1s adjacente pour générer un large fragment C2a qui demeure associé avec C4b. Le complexe résultant C4b2a est la C3 convertase de la voie classique du complément, ayant le rôle de lier et de cliver C3; la liaison à C3 est médiée par C4b et le clivage de C3 est effectué par C2a. Le clivage de C3 permet de générer C3b, et C3b forme des liens covalents avec la surface cellulaire adjacente ou à l'anticorps même. Une fois que C3b s'est déposé à la surface bactérienne, celui-ci peut lier le facteur B et générer de nouvelles C3 convertase via la voie alternative du complément, résultant ainsi en une boucle d'amplification. Ceci découle du fait qu'une seule C3 convertase entraîne le dépôt de centaines voire de milliers de molécules de C3b où le complément a été activé par un anticorps. Certaines des molécules C3b générées précédemment peuvent également s'associer avec la convertase et former le complexe C4b2a3b. Ce complexe sert alors de C5 convertase pour initier les étapes tardives de l'activation du complément qui mèneront à la formation du complexe d'attaque membranaire (191). Cependant, comme *S. suis* est une bactérie à Gram-positif, son épaisse couche de peptidoglycane prévient l'action bactéricide du complexe d'attaque membranaire (191, 222).

Enfin, pour mener à terme l'élimination de *S. suis* par opsonophagocytose suite à l'activation du complément, les bactéries opsonisées seront en mesure d'engager les récepteurs du complément. Le principal récepteur est le CR1, qui possède une forte affinité pour C3b et C4b (C3b > C4b) et est exprimé principalement par les cellules myéloïdes, notamment les neutrophiles, les monocytes, les macrophages et les lymphocytes T et B. Les phagocytes principalement utilisent CR1 pour lier et internaliser les particules opsonisées. La liaison de particules opsonisées par C3b et/ou C4b produit également des voies de signalisation intracellulaires permettant d'accroître les mécanismes microbicides des phagocytes, d'autant plus lorsqu'un FcγR est engagé simultanément (191, 222).

4. Vaccins glycoconjugués

L'histoire des vaccins à base de polysaccharides purifiés débutent dans les années 1930 avec la recherche effectuée sur les vaccins contre *S. pneumoniae*. Dès 1945, un premier vaccin anti-pneumocoque quadrivalent était disponible (223). Au même moment, l'arrivée des antibiotiques comme traitement des infections bactériennes a considérablement ralentie le développement de ce type de vaccin. Cependant, il est rapidement apparu que les traitements antimicrobiens, bien qu'efficaces, ne pouvaient être la seule solution si on considère les échecs de traitement, la fréquence de séquelles graves chez les individus récupérant de maladies comme la méningite, et l'émergence de souches antibiorésistantes. Dès 1978, un vaccin quadrivalent basés sur les CPS des sérogroupes A, C, W, et Y de *Neisseria meningitidis* a été approuvé, suivi du vaccin 23-valent contre *S. pneumoniae* en 1983 et du vaccin contre *Haemophilus influenzae* type b en 1985 (224).

Malgré ces développements prometteurs, les essais cliniques réalisés durant le développement des vaccins à base de polysaccharides purifiés ont démontrés que l'efficacité de ces vaccins chez les enfants âgés de moins de 2 ans est très faible. D'autant plus, cet échec vaccinal est d'autant plus préoccuppant que ce sont ces mêmes enfants qui sont le plus affectés par les maladies invasives causées par *H. influenzae*, *S. pneumoniae* et *N. meningitidis*. Ce fut l'élément déclencheur pour la recherche et le développement d'un nouveau type de vaccin, soit les vaccins glycoconjugués. Les premiers vaccins glycoconjugués rapportés pour immuniser contre *H. influenzae* type b, approuvés entre 1987 et 1990. Au même moment, la recherche était très active autant dans les milieux académiques et industriels pour développer des glycoconjugués contre les sérotypes les plus importants du point de vue épidémiologique contre *S. pneumoniae* et *N. meningitidis*. Celle-ci a mené à l'approbation et à la commercialisation de nombreux vaccins glycoconjugués depuis (224, 225).

Alors que les vaccins mentionnés précédemment étaient tous produits en utilisant des CPS purifiées de bactéries, il se faut de souligner le premier vaccin semi-synthétique contre *H. influenzae* type b a été développé et décrit par les Drs Verez-Bencomo et Roy en 2004, où la portion saccharidique a été produite par synthèse organique (226, 227). En plus d'assurer une

meilleure reproductibilité de lot en lot pour la CPS et l'absence complète de contaminants dérivés des bactéries, l'approche synthétique a permis de réduire considérablement les coûts de production du vaccin, permettant de rendre ce vaccin disponible dans les pays plus pauvres.

Il est également important de rappeler que tous les succès vaccinaux décrits précédemment ont été réalisés en médecine humaine. Malheureusement, les coûts de développement élevés pour ce type de vaccin ont freiné la recherche pour des applications en médecine vétérinaire (228). En 1992, un vaccin glycoconjugué à partir de CPS a été proposé pour protéger les porcs contre l'infection par *Actinobacillus pleuropneumoniae*, l'agent étiologique de la pleuropneumonie porcine (229-231). D'autres exemples de glycoconjugués ciblant les lipopolysaccharides (LPS) de pathogènes vétérinaires ont également été décrits (228).

4.1. Mécanisme d'action des vaccins glycoconjugués

Les CPS bactériennes, et les polysaccharides en général, sont des antigènes TI capables de stimuler directement la différentiation des lymphocytes B en plasmocytes (cellules sécrétrices d'anticorps) par le « cross-linking » des BCRs. Cependant, ce mécanisme n'est pas encore mature chez les enfants. De plus, la réponse TD est toujours plus efficace que la réponse TI. Dans un glycoconjugué, la protéine porteuse fournit les épitopes protéiques pour le recrutement des Th, alors que la CPS fournit les épitopes du polysaccharide pour l'engagement des cellules B CPS-spécifiques. Ainsi, lorsque la CPS du glycoconjugué lie le BCR et devient internalisée, les cellules B dégradent les antigènes protéiques et les présentent aux cellules T spécifiques à la protéine porteuse via le CMH-II. De plus, lors d'études récentes avec des glycoconjugués faits avec la CPS du sérotype III de GBS, un mécanisme additionnel a été proposé où le glycoconjugué peut être dégradé en couples glycane-peptides au sein des cellules B spécifiques pour la CPS. Les glycane-peptides résultant lient le CMH-II (par la portion du peptide) permettant à la portion glycane d'être présentée par les CPA pour engager le TCR de clones CPS-spécifiques de cellules T (Tcarb), qui à leur tour fournissent leur aide aux cellules B (208). Globalement, ces deux mécanismes permettent au vaccin glycoconjugué

d'induire une réponse TD aux polysaccharides et rend ces antigènes immunogènes dès un jeune âge (224).

En recevant l'aide des Th, les cellules B prolifèrent et se différencient en plasmocytes pour la production d'anticorps anti-CPS, avec la commutation isotypique principalement vers les IgG et la formation de cellules B mémoire. L'avidité des anticorps augmente également suite à la maturation d'affinité de la réaction germinale (224).

4.2. Protéines porteuses

Tel que mentionné précédemment, le rôle primaire de la protéine porteuse d'un glycoconjugué est de fournir les épitopes protéiques pour assurer une réponse TD pour un antigène autrement TI comme une CPS. Évidemment, ceux-ci remplissent très bien leur rôle si on se fie au succès de ces vaccins. Les cinq protéines porteuses traditionnelles et leurs détails sont présentés dans le **Tableau VI**.

Tableau VI. Protéines porteuses traditionnelles présentement utilisées dans les vaccins glycoconjugués commerciaux humains (224, 232).

Protéine	Toxoïde tétanique (TT)	Toxoïde diphtérique (DT)	« Cross-Reactive Material » 197 (CRM_{197})	« Outer Membrane Protein » de *Neisseria meningitidis* (OMP)	Protéine dérivée de *Haemophilus influenzae* non typable (PD)
Poids moléculaire	150 kDa	58 kDa	58 kDa	40 kDa	40 kDa
Provenance et production	*Clostridium tetani*	*Corynebacterium diphteriae*	*C. dipheriae* ou technologies d'ADN recombinant	*N. meningitidis* sérogroupe B	Technologie d'ADN recombinant
Détoxification	Formaldéhyde	Formaldéhyde	« Génétique »	Aucune	Aucune
Détails	Toxine tétanique détoxifiée	Toxine diphtérique détoxifiée	Mutant non toxique de la toxine diphtérique (G52E)		Lipoprotéine surface conservée chez toutes les souches d'*H. influenzae*

Initialement, les toxoïdes tétanique et diphtérique (TT et DT) furent choisis comme protéines porteuses pour les premiers vaccins en raison de leur production industrielle à grande échelle (vaccins contre le tétanos et la diphtérie), de leur grande immunogénicité et de leur innocuité démontrée sur plusieurs décennies de vaccination. Un des inconvénients avec ces toxoïdes est qu'ils doivent être détoxifiés par traitement au formaldéhyde, résultant en une altération des acides aminés disponibles pour la conjugaison et en une propension à oligomériser. De plus, la production à grande échelle de ces deux toxines (avant détoxification) comporte de nombreux risques pour la santé et la sécurité des travailleurs et du public général. Une alternative à la détoxification chimique de toxines est l'utilisation de la CRM_{197}, qui est un mutant détoxifié génétiquement (par mutation) de la toxine diphtérique, et est aujourd'hui largement utilisée dans la grande majorité des vaccins glycoconjugués (224, 232). Pour plus de détails sur quelle protéine porteuse est utilisée pour quel vaccin, on peut se référer à l'excellent article de synthèse suivant (233).

4.3. Méthodes chimiques de conjugaison

La préparation de bioconjugués est un domaine en développement constant de la chimie organique et bioorganique (234), ce qui rend difficile de suivre les tendances et les applications modernes. Le but premier de la chimie de conjugaison est de développer des méthodes simples et efficaces pour coupler une biomolécule à d'autres molécules. Ainsi, un prérequis est la modification préalable de la biomolécule à l'aide d'agents bifonctionnels. Ces réactifs peuvent être utilisés comme « linker », et peuvent même apporter de nouvelles fonctionnalités chimiques sur la molécule, ou simplement servir de « spacers » pour améliorer la disponibilité spatiale des groupes fonctionnels. Pour ce faire, la modification de la biomolécule requiert des fonctionnalités avec un excellent degré de sélectivité et de spécificité. Une bonne méthode de modification fonctionnelle devrait être généralement applicable à une large gamme de biomolécules. Cependant, encore aujourd'hui, aucune technique de couplage universelle n'a encore été développée (234, 235).

4.3.1. Méthodes pour la fonctionnalisation sélective de protéines

Généralement, la réactivité chimique des protéines dépend des acides aminés, de leurs chaînes latérales, de leurs résidus N- et C-terminaux et de leur structure. Du point de vue réactivité, les composantes cruciales des chaînes latérales des acides aminés sont le γ-carboxyle de l'acide glutamique, le β-carboxyle de l'acide aspartique, le thiol de la cystéine et l'ε-amine de la lysine. Parmi celles-ci, le thiol de la cystéine est le groupe nucléophile le plus puissant, suivi par l'amine et de l'hydroxyle. Étant donné que les groupes hydroxys ont un pKa similaire à celui de l'eau, ils sont généralement non- réactifs en solution aqueuse. L'imidazolyle de l'histidine, le thioéther de la méthionine, l'indolyle du tryptophane, le phénol de la tyrosine, le guanidyle de l'arginine et l'hydroxyle aliphatique de la sérine sont d'importance moindre (234, 235).

Le groupe thiol est un nucléophile doux qui peut être sélectivement modifié avec les bonnes conditions de réaction. De plus, les protéines diffèrent grandement dans le nombre de thiols qu'elles peuvent contenir : l'albumine sérique bovine (BSA) contient un seul résidu cystéine, alors que le « keyhole limpet hemocyanin » (KLH) en contient plus de 700 (236). Une stratégie possible pour générer des thiols est la réduction des ponts disulfures internes. Sinon, une autre stratégie consiste à dériver la protéine avec des « linkers » qui contiennent un thiol, et qui vont réagir avec une amine pour donner un nouveau thiol. Les dérivés réactifs de sulfhydryle les plus communs sont les composés α-halocarbonyles, les maléimides, les dérivés acryloyles, les aziridines, les oxyranes, les dérivés du fluorobenzène, les réactifs disulfure, etc. (236)

Le groupe ε-amine de la lysine diffère en pKa de l'α-amine primaire du N-terminal et est généralement le second groupe fonctionnel en termes de nombre chez les protéines. Parmi les réactions les plus importantes des amines, on retrouve l'acylation (formation d'amides avec des esters actifs), la réaction avec l'acide squarique et l'amination réductrice. D'autres réactifs comme les isothiocyanates, les isocyanates, les halogénures d'acyle, les azotures, les imidoesters, les anhydrides et les composés carbonate peuvent être employés pour dériver les amines (236).

Le carboxyle est le groupe fonctionnel le plus abondant chez les protéines. Ceux-ci peuvent donc être activés (pour un nucléophile comme l'amine) en tant qu'ester d'acyle, d'azoture d'acyle, d'acylimidazoles, d'anhydrides, etc. Bien qu'il y ait plusieurs façons de faire, deux approches sont communément utilisées pour coupler un dérivé réactif de carboxyle avec une amine pour former un amide. Pour la première méthode, un agent réactif acylant est formé à partir de l'acide suivi du traitement immédiat par l'amine. La seconde méthode consiste à le générer *in situ* en présence de l'amine à l'aide d'un agent activateur ou de couplage. L'activation des groupes carboxyles des protéines peut mener à du « cross-linking » intra- ou intermoléculaire, surtout lors de l'emploi de la première méthode. Également, la succinylation des groupes amino mène non seulement à un nombre accru de groupes carboxyliques, mais peut également permettre d'améliorer les propriétés immunologiques des protéines (ou de leurs conjugués finaux) (237).

4.3.2. Méthodes pour la fonctionnalisation sélective des sucres

Les glycanes, dont les polysaccharides font partie, possèdent une très grande diversité compositionelle (monosaccharides) et structurale (liaisons). Les monosaccharides peuvent également être acétylés, phosphorylés ou branchés. Exception faite des hydroxyles, les monosaccharides portent souvent des groupes réactifs, comme notamment des carboxyles et des amines (234, 235).

Pour ce qui est des polysaccharides, dans presque tous les cas, ceux-ci doivent être dérivés chimiquement avant de pouvoir procéder à la conjugaison. Les méthodes les plus couramment employées consistent en l'oxydation de diols vicinaux au periodate, l'oxydation des *sec*-hydroxyles par la 1,1,1-triacétoxy-1,1-dihydro-1,2-benzodoxol-3(1H)-one ou le periodinane de Dess-Martin pour produire des aldéhydes ou à l'aide de d'oxydes d'azote pour produire des groupes carboxyles (234, 235). Un autre type de modification consiste à « activer » les hydroxyles par le bromure de cyanogène ou le tétrafluoroborate de 1-cyano-4-(diméthylamino)pyridinium, qui produira des dérivés réactifs cyanato, ainsi que la réaction

d'Ugi à multiples composantes. L'hydrolyse acide des polysaccharides est utilisée pour obtenir plus des saccharides de plus petites tailles afin de faciliter les réactions de couplage.

4.3.3. Méthodes de couplage « classiques »

Parmi toutes les méthodes de conjugaison dites « classiques », visant notamment la formation de liens amides, thioéthers ou l'activation d'hydroxyles, nous allons surtout nous concentrer sur l'amination réductrice dans le cadre de cette thèse. Il n'en demeure pas moins que tout un arsenal de fonctions, de modifications et de réactifs possibles est à la disposition du chimiste pour réussir sa conjugaison.

4.3.3.1 Amination réductrice

L'amination réductrice est une des premières méthodes qui a été utilisée pour la préparation des glycoconjugués et une des plus populaires encore à ce jour, spécialement à partir de mono- et oligosaccharides libres non protégés. Les aldéhydes et les cétones libres réagissent avec les amines (également avec les hydrazines, les hydrazides et les dérivés oxime) pour former une imine instable (réaction réversible) nommée base de Schiff. Après réduction de l'imine formée initialement, des amines secondaires stables sont obtenues. Cette réaction en deux étapes est donc l'amination réductrice. L'imine formée initialement (toujours en équilibre avec le carbonyle et l'amine du départ) est convertie vers une amine secondaire stable à l'aide d'un agent réducteur, habituellement à l'aide d'une réaction « one pot ». Comme agents réducteurs, les hydroborates, dont le $NaBH_3CN$ est le plus communément utilisé, sont généralement employés pour leur grande sélectivité envers les imines et leur non-réactivité relative envers les autres groupes (234, 235).

Si un sucre ne possède aucun groupe aldéhyde libre, un « linker » ou « spacer » avec une fonction carbonyle protégée doit être employé pour obtenir un aldéhyde comme partenaire fonctionnel. Dans le cas des polysaccharides, dont la CPS du sérotype 2 de *S. suis*, il faut effectuer une oxydation de groupes hydroxyles vicinaux pour introduire la fonction aldéhyde nécessaire. Malheureusement, cette approche d'oxydation, qui est généralement non sélective,

va produire une distribution aléatoire d'aldéhydes et peut endommager les épitopes de la molécule (235). Ceci étant dit, dans les cas d'amination réductrice avec la CPS du sérotype 2 de *S. suis* comme avec les CPS de GBS, elles possèdent toutes un acide sialique, soit un sucre à 9 carbones dont les 3 derniers forment une chaîne glycérol dont la conformation est libre (aucune contrainte de rotation comme dans le cas d'une conformation chaise ou bâteau) (voir **Figures 2 et 6**). Ainsi, lors de l'oxydation au periodate, cette chaîne glycérol sera sélectivement clivée entre les carbones C8–C9 (ou plus rarement entre les carbones C7–C8) pour produire l'aldéhyde requis pour la conjugaison (238, 239). De plus, en contrôlant attentivement la quantité de periodate dans la réaction, il est possible d'oxyder seulement une minorité des acides sialiques, ce qui assure que la majorité des épitopes demeurent intacts.

Le mécanisme de l'amination réductrice, appliqué à la CPS du sérotype 2 de *S. suis*, est donc présenté à la **Figure 6**.

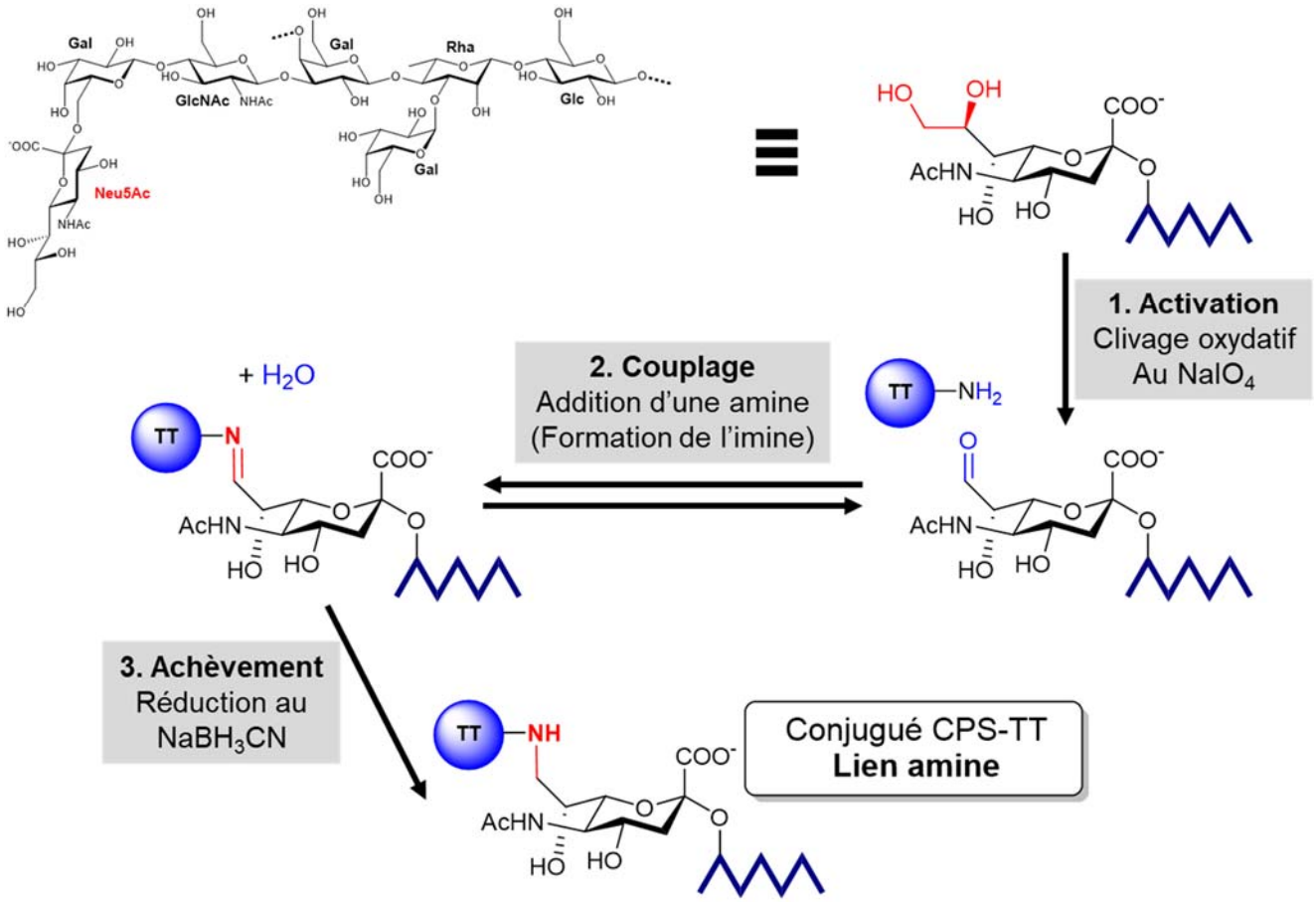


Figure 6. Mécanisme chimique de la réaction d'amination réductrice entre la CPS du sérotype 2 de *S. suis* et le toxoïde tétanique (TT) (236).

Tout d'abord, l'acide sialique de la CPS est oxydé par le periodate, générant un groupe aldéhyde pouvant être utilisé par la conjugaison au TT. Lors de l'addition du TT, les amines libres de la protéine vont réagir avec les aldéhydes de la CPS pour former une base de Schiff (imine instable). Finalement, l'addition d'un agent réducteur comme le cyanoborohydrure va réduire l'imine en une amine secondaire stable. C'est ainsi que l'amination réductrice permet de coupler la CPS du sérotype 2 à une protéine porteuse pour l'obtention d'un vaccin glycoconjugué.

4.4. Adjuvants

Les adjuvants désignent toutes substances capables d'augmenter l'intensité de la réponse immune dirigée contre un antigène. Il en existe de nombreuses variétés, de natures et d'origines différentes. Deux types d'adjuvants se distinguent, ils peuvent être des immunostimulants ou des « véhicules ». Les immunostimulants activent directement les cellules immunes en se liant à des récepteurs, tels que les TLRs, des cellules immunes. Ainsi, ils influencent directement le développement la réponse immunitaire de type 1 ou de type 2. Les « véhicules » contiennent eux-mêmes même l'antigène et déterminent la façon dont il sera présenté aux cellules immunes. Ils contiennent aussi une capacité immunostimulante, car ils sont constitués de corps étrangers (240, 241).

La plupart du temps, les adjuvants sont indispensables au développement de la réponse immune lors de la vaccination. En effet, le pouvoir immunogène d'un vaccin non adjuvanté est souvent trop faible ; même s'il contient les épitopes protecteurs adéquats, il manque un agent capable d'amplifier et d'orienter la réponse immune spécifique (240, 241). Par exemple. les adjuvants sont indispensables dans la formulation des vaccins sous-unitaires. Malgré que ce genre de vaccin reste le plus sécuritaire, il est cependant moins immunogène de par l'utilisation de protéines/peptides solubles comme antigène. Le choix de l'adjuvant est crucial pour le développement d'un vaccin en médecine vétérinaire et humaine. En effet, ces substances influencent la magnitude et la qualité de la réponse adaptative en agissant directement ou indirectement sur la commutation isotyique des lymphocytes B, afin de garantir la meilleure protection contre le pathogène. Ils doivent être stables, sécuritaires et faciles à utiliser, et permettre une diminution de l'utilisation de l'antigène. Un adjuvant n'est pas universel pour la formulation d'un vaccin, il en existe de nombreux pouvant être utilisés en médecine vétérinaire. Ils agissent par différents mécanismes, et leur mode d'action pour déclencher la réponse immunitaire est parfois incomplètement connu (240, 241). Les principales catégories d'adjuvants utilisés en médecine vétérinaire seront décrites ci-dessous.

4.4.1. Les constituants bactériens

Des adjuvants contenant des constituants bactériens ou des préparations de paroi bactériennes sont des adjuvants capables de mimer les signaux pro-inflammatoires reçus par le système immunitaire lors des interactions entre les PAMPs et les PRRs.

4.4.1.1. Adjuvant complet de Freund

Le (FCA) est bien connu. Alors qu'il est aussi un adjuvant faisant partie de la catégorie des émulsions eau-huile, celui-ci est constitué d'une mycobactérie inactivée (*Mycobacterium tuberculosis*) en plus de son émulsion eau-dans-huile. Le FCA combine l'effet dépôt (expliqué dans la partie des adjuvants de type émulsion eau-huile) d'une émulsion eau-dans-huile et la capacité d'activer une réponse immunitaire spécifique par la présence de la mycobactérie inactivée. En effet, le FCA cible les TLR2, TLR3 et TLR4 des cellules immunes et induit une forte réponse immunitaire. Il a beaucoup été utilisé dans des essais de vaccination contre *S. suis*. La majorité de ces essais vaccinaux se sont révélés efficaces, grâce au fort pouvoir immunogène de cet adjuvant. Or, le FCA est connu pour induire des effets secondaires considérables chez l'animal vacciné. En effet, il entraîne l'apparition d'abcès, de granulomes et de nécroses cutanées. C'est aussi un agent pyrogène et peut provoquer des polyarthrites et diverses lésions liées à des réactions auto-immunes. Le FCA ne peut être administré qu'une seule fois; des injections répétées peuvent provoquer une réaction anaphylactique fatale. Pour les rappels, il est recommandé d'utiliser l'adjuvant incomplet de Freund (IFA). Le FCA a été retiré du marché dans la majorité des pays de par ces forts effets secondaires engendrés; cependant une minorité de pays l'utilise encore, telle que la Chine (240, 241).

4.4.1.2. « CpG oligodeoxynucleotides »

Des ADN bactériens sont employés comme adjuvant, tels que les « CpG oligodeoxynucleotides » (CpG ODN) qui miment l'ADN bactérien. Ce sont des ADN simples brins synthétiques contenant des motifs CpG non méthylés. Ils se lient aux TLR9 des cellules et activent le système immunitaire. Ils améliorent la présentation de l'antigène et stimulent les réponses immunes cellulaires et humorales. Il existe trois classes de CpG ODN basés sur leurs

caractéristiques structurales et sur leurs activités au niveau des cellules immunes. Les CpG ODN de classe A induisent la production de IFN-α par l'activation des DCs plasmacytoïdes, mais sont généralement de faibles activateurs de la production des cytokines pro-inflammatoires, alors que les CpG ODN de classe B, activent les cellules B et des autres cellules exprimant le TLR9 et induisent la production de cytokines pro-inflammatoires, mais n'activent que faiblement les pDC. Enfin les CpG ODN de classe C combinent l'activité des deux dernières classes. Les CpG sont utilisés en phase clinique pour la vaccination des humains contre la malaria, l'hépatite B ainsi que le virus de l'influenza (242). Chez le porc, les CpG ont été utilisés dans des essais de vaccination contre des virus, tel que le virus pseudorabies (243).

4.4.2. Émulsions eau-huile

Les adjuvants de type émulsion eau-huile regroupent différents types d'émulsion. Une émulsion est un système où deux phases normalement non miscibles sont dispersées l'une dans l'autre et stabilisées par des agents tensioactifs. Deux sortes d'émulsions peuvent être utilisées comme adjuvant, à savoir les émulsions eau-dans-huile (w/o pour « water-in-oil ») et huile-dans-eau (o/w pour « oil-in-water »). La combinaison de ces deux émulsions peut aussi être retrouvée en vaccinologie (émulsion eau-dans-huile-dans-eau) (240, 241). Les huiles peuvent être de différentes origines, telles qu'animale, végétale ou minérale, et peuvent influencer l'efficacité de l'adjuvant selon leur composition. En effet, l'huile animale est composée de squalène qui se décrit comme un lipide hydrocarboné naturellement produit par les organismes supérieurs. C'est un intermédiaire essentiel dans le métabolisme du cholestérol, des hormones stéroïdes et de la vitamine D chez l'homme (244). Lorsque ces huiles animales font partie d'un vaccin, elles sont par conséquent plus tolérées par l'organisme (245). D'ailleurs, deux adjuvants composés d'huile animale à base de squalène sont actuellement brevetés pour l'utilisation dans la vaccinologie humaine (l'adjuvant AS03®, et l'adjuvant MF59®) (246). Néanmoins, les huiles animales restent moins efficaces que les huiles minérales pour la composition de vaccins. En effet, ces dernières sont composées en majorité de MARCOL 52, ou encore appelée l'huile blanche qui est dérivée du pétrole (240). Ce type d'huile n'est donc pas métabolisé par l'organisme et par conséquent, induit des effets

indésirables au site d'injection, mais demeure efficace pour activer le système immunitaire (240).

Le mécanisme d'action des adjuvants de type émulsion eau-huile se déroule par la formation d'un dépôt au site d'injection. L'effet dépôt consiste à limiter une rapide élimination de l'antigène par l'hôte en empêchant sa dilution et sa dégradation. Grâce à un relâchement lent de l'antigène intact, ce type d'adjuvant permet une exposition lente et prolongée de l'antigène aux cellules immunitaires. Cette exposition prolongée induit une stimulation continue des cellules B, et résulte dans une production élevée d'anticorps par l'hôte (240). En général, ces adjuvants sont trop toxiques pour être utilisés comme vaccin prophylactique chez l'humain, bien qu'ils puissent convenir pour certaines conditions, telles que le cancer terminal où il y a une plus grande tolérance des effets secondaires. Les effets secondaires de cette catégorie d'adjuvant peuvent se décrire généralement par des réactions inflammatoires, des granulomes et des ulcères qui apparaissent au site d'injection (247).

4.4.2.1. Émulsions eau-dans-huile (w/o)

L'émulsion w/o est représentée par des gouttes de solution aqueuse d'antigène hydrophile d'un diamètre de l'ordre du nanomètre, englobées dans une phase lipophile continue. Cette formulation permet la libération progressive de l'antigène au niveau du site d'injection et sa présentation sur une surface étendue tout en protégeant la dégradation de l'antigène. En outre, elle exerce un effet de « corps étranger » par lequel elle attire les cellules de l'immunité et provoque ainsi une réaction inflammatoire (240, 241).

4.4.2.1.1. Adjuvant incomplet de Freund

L'IFA est un adjuvant communément utilisé en recherche, cependant il engendre de forts effets secondaires. Il est préparé à partir d'huiles non métabolisables (huile de paraffine et un émulsifiant). Il ne contient pas de *M. tuberculosis* comme retrouvé dans le FCA, il est donc moins inflammatoire, mais provoque des lésions au site d'injection et est potentiellement cancérigène. L'IFA induit le développement d'une forte réponse immunitaire, mais est

incapable de stimuler les types de réponses cellulaires efficaces contre les tumeurs et contre de nombreuses infections virales. L'intérêt croissant pour le bien-être des animaux de laboratoire a favorisé le développement d'adjuvants équivalents à l'IFA, mais ayant un niveau de toxicité acceptable (240, 241).

4.4.2.1.2. TiterMax® et TiterMax Gold®

Les adjuvants de type TiterMax® sont des émulsions w/o composées d'un copolymère synthétique et du squalène. Le TiterMax® est un adjuvant beaucoup moins toxique que le FCA et l'IFA, mais conserve le même taux de production d'anticorps démontré chez la souris et le hamster (240). Cependant, le TiterMax® peut causer des dommages tissulaires au niveau de son site d'injection. La version Gold (TiterMax Gold®) induit des taux d'anticorps particulièrement élevés et persistants. Le TiterMax Gold® est constitué d'une huile de squalène métabolisable, du sorbitan monooléate 80 comme émulsifiant, d'un copolymère (CRL8300) et de microparticules de silice. Ces adjuvants sont essentiellement utilisés pour la recherche en vaccinologie (240).

4.4.2.1.3. STIMUNE®

Le STIMUNE® (ou encore nommé Specol) est composé d'une huile minérale appelée MARCOL 52, ainsi que des émulsifiants tels que le Span 85 et le Tween 85. Le STIMUNE® induit une forte réponse anticorps comparable à celle obtenue avec le FCA chez le lapin, la souris, le porc et des perruches. Cet adjuvant peut induire des effets secondaires tels que la fièvre, mais aussi des lésions au site d'injection (240). Le STIMUNE® est souvent utilisé dans des essais de vaccination chez le porc, notamment contre *S. suis* avec des bactérines (133).

4.4.2.2. Émulsions huile-dans-eau (o/w)

L'émulsion o/w est représentée par des préparations de copolymères de chaînes hydrophiles de polyoxyéthylène et de chaînes hydrophobes de polyoxypropylène incorporées dans une huile. Ce type de structure particulaire favorise le captage de l'antigène par les CPAs. Lors de l'administration d'une émulsion o/w, la phase aqueuse se disperse rapidement

et les sphérules d'huile libérées transportent l'antigène directement vers les ganglions via les vaisseaux lymphatiques. Contrairement aux émulsions w/o, la dispersion rapide d'une émulsion o/w permet supposément d'éviter la formation de granulomes et d'abcès (240, 241).

4.4.2.2.1. Montanide ISA®

Les adjuvants de la série Montanide ISA®, commercialisés par la compagnie SEPPIC, sont utilisés en médecine vétérinaire. SEPPIC propose une série d'émulsions o/w ou w/o prêtes à être utilisées sur plusieurs espèces animales tels que les bovins, les ovins, les chevaux, les poissons, la volaille ainsi que le porc. Parmi eux, l'adjuvant Montanide ISA 25 VG® est une émulsion o/w composée d'une huile minérale connue pour être efficace, mais induit des réactions locales au site d'injection (248). Le Montanide ISA 25 VG® est particulièrement utilisé pour vacciner les ovins et les porcs et est capable d'améliorer l'efficacité d'un vaccin via l'induction d'une forte réponse immunitaire à court terme. Le Montanide ISA 25 VG® a fait l'objet de formulation de bactérines contenant des cellules entières de *S. suis* afin de vacciner les porcs contre *S. suis* du sérotype 2. La formulation vaccinale a révélé une diminution de la bactériémie et de l'apparition des signes cliniques (méningite, pneumonie) ainsi qu'une diminution de la mortalité par rapport aux animaux non vaccinés, suite à une infection par *S. suis* (138). Selon SEPPIC, le Montanide 25 VG® peut induire des effets néfastes chez le porc, tel que l'induction d'une forte fièvre.

Afin d'éliminer ces effets indésirables, d'autres adjuvants Montanide® ont été développés. Le Montanide ISA 35 VG®, composé d'une huile métabolisable ainsi que du mannitol oléate comme émulsifiant, est recommandé pour la vaccination des chevaux et des porcs.

4.4.2.3. Émulsions eau-dans-huile-dans-eau (w/o/w)

Les émulsions w/o/w sont des émulsions en phase aqueuse continue dans lesquelles les gouttelettes d'huile contiennent une phase aqueuse secondaire (double émulsion). En raison de leur structure, les émulsions w/o/w peuvent induire une réponse immunitaire offrant une protection à court et à long terme. Des essais sur le terrain ont démontré que ces adjuvants

peuvent stimuler les réponses immunitaires aussi bien humorales que cellulaires. En bref, ce type d'adjuvant permet de combiner la forte immunogénicité des émulsions w/o avec la fluidité des émulsions o/w. SEPPIC produisent notamment le Montanide ISA 201 VG® qui est composé d'huile minérale et de mannitol oléate comme émulsifiant (240).

4.4.3. Saponines

Les saponines sont des glycosides naturels de stéroïdes ou triterpéniques qui, en plus d'être utilisés dans divers secteurs industriels comme détergents ou émulsifiants, présentent de nombreuses activités biologiques et pharmacologiques différentes, dont notamment l'activation du système immunitaire des mammifères. Des extraits de saponines bruts à partir de l'écorce de *Quillaja saponaria* Molina (un arbre d'Amérique du Sud) ont été premièrement utilisés comme adjuvant, cependant ces extraits hétérogènes et aux effets peu prévisibles ont aujourd'hui fait place à des préparations standardisées, telles que le Quil-A® et ses dérivées (comme le QS-21®). Leur efficacité a été évaluée dans de nombreux essais cliniques. Leur capacité à stimuler à la fois la réponse immunitaire humorale et cellulaire, rend ces adjuvants idéaux pour une utilisation dans les vaccins sous-unitaires dirigés contre des antigènes exogènes, contre les pathogènes intracellulaires, ainsi que des vaccins thérapeutiques contre le cancer. Cependant, des effets indésirables peuvent survenir tels qu'une toxicité élevée ou une activité hémolytique. En effet, les aponines interagissent avec le cholestérol des membranes cellulaires et forment des pores au sein de ces dernières. De par ces effets toxiques, les saponines sont restreintes aux usages dans la vaccinologie animale (241, 249).

Le mécanisme d'action des saponines sur la stimulation du système immunitaire n'est pas clairement compris, mais de nombreuses explications ont été avancées. Il est probable que les saponines interagissent avec les CPAs pour induire la production de cytokines. L'incorporation des saponines dans les membranes cellulaires ou les endosomes des cellules immunitaires exposerait ainsi l'antigène aux protéases cytosoliques. Comme évoqué précédemment, les saponines forment des pores à travers les membranes cellulaires en s'intercalant entre le cholestérol et les lipides membranaires, et ainsi les antigènes auraient

directement accès à la voie endogène de la présentation des antigènes aux lymphocytes T CD8+ (250).

4.4.3.1. Quil-A®

L'adjuvant Quil-A® a été conçu en 1978 par Dalsgaard, à partir de mélange enrichi de saponine extraite de l'arbre *Quillaja saponaria* Molina (249). Le Quil-A® induit à la fois une réponse immunitaire humorale et cellulaire, et produit des isotypes d'Ig variés. Cet adjuvant est largement utilisé en médecine vétérinaire, notamment dans le vaccin prévenant la maladie « foot-and-mouth » en médecine bovine, ainsi que dans la recherche vaccinale contre *S. suis* chez le porc et la souris (140, 144).

4.4.4. Sels minéraux (Alum)

Les composants à base d'aluminium, particulièrement le phosphate d'aluminium et l'hydroxyde d'aluminium (généralement nommés « Alum ») sont communément utilisés dans les préparations vaccinales pour la médecine humaine et vétérinaire. Les vaccins composés d'aluminium sont sécuritaires et efficaces, et sont utilisés depuis plus de 60 ans (240). Néanmoins, ils manifestent certains désavantages. En effet, les antigènes adjuvantés à l'Alum sont rapidement éliminés après l'injection, et la production d'anticorps reste modérée (un pic de production d'anticorps à 3–4 semaines suivi d'un déclin rapide), et par conséquent, des rappels sont nécessaires (240). Ainsi, l'intérêt de l'Alum réside essentiellement dans sa sécurité (240, 241). Au contraire des autres catégories d'adjuvants, le mode d'action de l'Alum a largement été étudié. Il se présente sous forme de précipités insolubles de type gel sur lesquels s'adsorbe l'antigène par des forces électrostatiques et fournit un effet dépôt à court terme. L'antigène est concentré localement, une fois injecté.

Pour ce qui est des voies d'activation de l'Alum, celui-ci activerait les voies des inflammasomes. L'inflammasome est un complexe protéique oligomérique impliqué dans l'immunité innée. Il est constitué de plusieurs protéines qui s'assemblent à la suite de la reconnaissance de divers signaux inflammatoires par des récepteurs spécifiques tels que le «

NOD-like receptor protein 3 » (NLRP3). L'inflammasome, une fois assemblé, favorise la production des cytokines pro-inflammatoire. L'Alum ciblerait donc le NLRP3 et activerait l'assemblage de l'inflammasome dont cette activité procurerait son pouvoir adjuvant (251). Il existe plusieurs préparations d'adjuvants composés d'Alum et utilisés dans des formulations des vaccins pour la médecine humaine, tels que l'aluminium potassium sulfate [$AlK(SO_4)_2$], l'aluminium hydroxide [$Al(OH)_3$] appelé Alhydrogel® et l'aluminium phosphate [$Al(PO_4)_3$] (251). L'Alhydrogel® a été élu comme la préparation standard internationale pour les gels d'hydroxyde d'aluminium hydroxyde. L'Alhydrogel® est présent dans de multiples formulations de vaccins commerciaux (251). L'Alum a aussi fait l'objet de plusieurs études de vaccination contre *S. suis* à la fois chez le porc et chez la souris (133).

Ainsi, basé sur cette recension de la littérature, l'**hypothèse générale** de cette thèse est que les anticorps anti-CPS protègent l'hôte en permettant l'élimination de *S. suis*, un pathogène extracellulaire encapsulé, par opsonophagocytose. Plus spécifiquement :

i. Ces anticorps sont également protecteurs contre toute souche d'un même sérotype, surmontant ainsi la grande variation phénotypique parmi celles-ci.
ii. Les CPS de *S. suis* sont des candidats idéaux pour le développement de vaccins efficaces pour la prévention de cette maladie.
iii. Ces CPS n'ont pas toutes les mêmes propriétés immunogènes, compte tenu de leurs différences compositionnelles et structurales.
iv. La création et l'emploi d'un vaccin glycoconjugué préparé à partir d'une CPS renversera son caractère non immunogène par la génération d'une réponse TD.

Les **objectifs généraux** de cette thèse sont, d'abord, de poursuivre la caractérisation des structures des CPS de *S. suis*, puis d'étudier les interactions entre celles-ci et le système immunitaire de l'hôte dans le cadre du développement d'une réponse humorale protectrice.

Ainsi, les **objectifs spécifiques** se déclinent selon les deux grands axes de recherche suivants :

AXE I – Diversité des capsules polysaccharidiques de *S. suis*

1. Recenser les sérotypes de *S. suis* impliqués lors d'infections chez le porc et chez l'homme dans la littérature.

2. Déterminer les structures des CPS pour les sérotypes importants de *S. suis* par une approche de chimie structurale (analyses chimiques et spectroscopiques).

AXE II – Réponse immunitaire humorale face aux capsules polysaccharidiques de *S. suis*

3. Caractériser l'antigénicité des CPS de *S. suis* dont la structure est connue et d'évaluer leur immunogénicité en évaluant la réponse humorale anti-CPS. Pour ce faire, les sous-objectifs suivants ont été établis :
   3.1. Caractériser l'antigénicité de ces CPS purifiées en évaluant les éléments structuraux reconnus par les sérums de sérotypie à l'aide de techniques sérologiques.
   3.2. Évaluer le pouvoir activateur de ces CPS purifiées face à des DCs par des stimulations *in vitro* en caractérisant la réponse inflammatoire et le rôle des TLR.
   3.3. Évaluer l'immunogénicité de ces CPS en caractérisant la nature et la fonctionnalité de la réponse humorale anti-CPS suite à des immunisations *in vivo* à l'aide de CPS purifiées, de bactéries entières tuées et de bactéries vivantes (infections à dose sous-létale).

4. Créer un prototype de vaccin glycoconjugué dirigé contre le sérotype 2 de *S. suis* et à en faire la preuve de concept chez le porc (volet appliqué) en caractérisant la nature et la protection de la réponse humorale anti-CPS suite à une immunisation *in vivo*.

5. Évaluer l'immunogénicité d'un vaccin glycoconjugué contre le sérotype 2 de *S. suis* et la fonctionnalité des anticorps produits, ainsi que l'influence du modèle expérimental sur ceux-ci. Pour ce faire, les sous-objectifs suivants ont été établis :
   5.1. Caractériser la nature et la fonctionnalité de la réponse humorale induite par un vaccin glycoconjugué dirigé contre la CPS du sérotype 2 de *S. suis* suite à des immunisations *in vivo*.
   5.2. Étudier l'influence du choix de l'adjuvant et du modèle animal sur l'immunogénicité du glycoconjugué en caractérisant la nature et la fonctionnalité de la réponse humorale anti-CPS.
   5.3. Obtenir des anticorps monoclonaux anti-CPS obtenus grâce au glycoconjugué, puis étudier leur spécificité et leur potentiel protecteur par l'emploi de techniques sérologiques et d'essais fonctionnels *in vitro* et *in vivo*.

III. MÉTHODOLOGIE ET RÉSULTATS

ARTICLE I

Streptococcus suis, an important pig pathogen and emerging zoonotic agent – An update on the worldwide distribution based on serotyping and sequence typing

---

Guillaume Goyette-Desjardins [1$], Jean-Philippe Auger [1$], Jianguo Xu [2], Mariela Segura [1], and Marcelo Gottschalk [1]

[$]These authors contributed equally to the manuscript

[1] Groupe de recherche sur les maladies infectieuses en production animale, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, Quebec, Canada;
[2] State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China

Publié dans *Emerging Microbes and Infections.* **2014**. 3(6):e45.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis co-premier auteur de cet article de synthèse. J'ai participé activement à la recension de la littérature, aux analyses et à l'écriture de l'article pour les parties concernant les sérotypes (50%).

Abstract

Streptococcus suis is an important pathogen causing economic problems in the pig industry. Moreover, it is a zoonotic agent causing severe infections to people in close contact with infected pigs or pork-derived products. Although considered sporadic in the past, human *S. suis* infections have been reported during the last 45 years, with two large outbreaks recorded in China. In fact, the number of reported human cases has significantly increased in recent years. In this review, we present the worldwide distribution of serotypes and sequence types (STs), as determined by multilocus sequence typing, for pigs (between 2002 and 2013) and humans (between 1968 and 2013). The methods employed for *S. suis* identification and typing, the current epidemiological knowledge regarding serotypes and STs and the zoonotic potential of *S. suis* are discussed. Increased awareness of *S. suis* in both human and veterinary diagnostic laboratories and further establishment of typing methods will contribute to our knowledge of this pathogen, especially in regions where complete and/or recent data is lacking. More research is required to understand differences in virulence that occur among *S. suis* strains and if these differences can be associated with specific serotypes or STs.

1. Introduction

Streptococcus suis is one of the most important pathogens in the porcine industry causing septicemia, meningitis and many other infections. In addition, it is an emerging zoonotic agent responsible for septicemia with or without septic shock, meningitis and other less common infections in humans.(1) During the last decade, the number of reported human cases due to *S. suis* has dramatically increased, and while most sporadic human cases of infection appear to be due to close occupational contact with pigs/pork products, particularly in Western countries (farmers, veterinarians, butchers, food processing workers, etc.), two epidemics were recorded in China in 1998 and 2005.(1) As of 2006, the number of human cases reported in Asia has increased.(2, 3) In fact, in some Asian countries, the general population is at risk.(2) However, an update on the distribution of the different serotypes and sequence types (STs), as determined by multilocus sequence typing (MLST), of strains responsible for infections in both pigs and humans from around the world have not been recently compiled. Yet, knowledge of this distribution is necessary in order to not only understand the current situation regarding *S. suis*, but also to evaluate areas where knowledge is lacking. This review covers the global distribution of the *S. suis* serotypes and STs responsible for infections reported in pigs from January 1st, 2002 to December 31st, 2013. A complete review on serotypes from humans since the first description in 1968 was also carried out. However, data of the STs from human cases are available only since 2002. Complete reviews have already covered the different virulence factors implicated in the pathogenesis of the infection caused by this important pathogen(4, 5), and this will not be further addressed in this review.

1.1 Brief description of the general aspects of infection in pigs and human

The natural habitat of *S. suis* is the upper respiratory tract of pigs, more particularly the tonsils and nasal cavities, but also the genital and digestive tracts.(6) With almost 100% of pig farms worldwide having carrier animals, *S. suis* is one of the most important bacterial pig pathogens. Transmission of *S. suis* among animals is considered to be mainly through the respiratory route.(6) Of the various manifestations of the disease, septicemia and meningitis are by far the most striking features, but endocarditis, pneumonia and arthritis can also be

observed.(7) Nevertheless, in peracute cases of infection, pigs are often found dead with no premonitory signs of disease.(7) Presumptive diagnosis of infection in pigs is usually based on clinical signs and macroscopic lesions.(8) Confirmation of the infection is mandatory and must be achieved by isolation and characterization of the pathogen.(8)

Since first described in Denmark in 1968(9), over 1 600 human cases of *S. suis* infection have been reported with many more probably never diagnosed or misdiagnosed. *S. suis* is the most common cause of adult meningitis in Vietnam, the second most common in Thailand, and the third most frequent cause of community-acquired bacterial meningitis in Hong Kong.(10-12) Different from pigs, the main route of entry of *S. suis* in humans is thought to be through contact of cutaneous lesions, most usually on the hands and arms, with contaminated animals, carcasses or meat.(3) However, this situation seems to be different in some Asian countries where the oral route is taken into consideration since many cases of infection have been reported after ingestion of contaminated raw pork products.(13) In Western countries, infections in humans most usually occur sporadically.(1) After an incubation period that ranges from a few hours to days(13), *S. suis* usually produces meningitis in humans, but is also responsible for cases of endocarditis, pneumonia, peritonitis, arthritis, and other less common diseases usually related to generalized septicemia.(14, 15) In addition, peracute infections with shock and a high mortality rate have been described, particularly in the case of the streptococcal toxic shock-like syndrome (STSLS) closely associated with the 2005 Chinese epidemic, but also observed independently of this outbreak.(16)

2. Identification of *S. suis*

S. suis is an encapsulated Gram-positive bacterial coccus that occurs singly, frequently in pairs, or occasionally in short chains. The organism grows well in aerobiosis, but growth is enhanced by microaerophilic conditions. The majority of strains are alpha-hemolytic on bovine and sheep blood agar plates after 24 hours of incubation at 37ºC.

2.1 Pig and human strains isolated from clinical cases of infection

For clinical cases in pigs, isolation and identification of strains is relatively easy since successful identification can be achieved by a minimum of biochemical tests and confirmed by serotyping (see below).(17) However, the use of rapid multi-test biochemical kits may be misleading as some strains of *S. suis* can be misidentified.(18) Although *S. suis* field isolates readily grow on media used for culturing meningitis-causing bacteria and veterinary diagnostic laboratories easily identify this pathogen, many human diagnostic laboratories are less aware of this pathogen and may misidentify it as enterococci, *S. pneumoniae*, *Streptococcus bovis*, viridans group streptococci (e.g. *Streptococcus anginosus* and *Streptococcus vestibularis*) or even *Listeria monocytogenes*.(14, 18-22) In many cases, the initial Gram stain presumptive diagnosis of the cerebrospinal fluid (CSF) specimen is pneumococcal meningitis. This confusion may have led to the misdiagnosis of *S. suis* infections in the past. Many cases were diagnosed retrospectively after the isolates were initially misidentified.(23) More recently, PCR tests have been used to directly detect *S. suis* DNA from CSF samples with a sensitivity considerably higher than direct culture, especially if antibiotics have been used.(2) However, most PCR tests used for humans detect serotype 2 strains only and will not detect infections caused by other *S. suis* serotypes (see below). Instead of the serotype 2 PCR test, it is advisable to use other validated PCR tests that allow the detection of the *S. suis* species (see below).

2.2 Strains isolated from clinically healthy pigs

S. suis is a normal inhabitant of the oral cavity of pigs. The biochemical identification of isolates recovered from clinically healthy pigs (mainly from tonsil samples) is difficult to achieve due to the presence of other streptococci that are part of the normal oral microflora and that are phenotypically similar to *S. suis*. For this reason, molecular biology techniques have been developed during the last decade to allow the detection and identification of *S. suis* strains, such as a PCR assay targeting the specific house-keeping gene encoding for the glutamate dehydrogenase (*gdh*).(24) Although widely used, the *gdh* PCR test can sometimes fail to correctly identify certain *S. suis* isolates.(25) Using this test, it has also been reported that there was a risk of misidentifying *Streptococcus gallolyticus* as *S. suis*.(26)

3. Serotyping

3.1 Definition of serotypes

Serotyping, which should be a part of the routine identification of *S. suis* strains recovered from diseased pigs and humans, will provide further confirmation regarding the pathogen's identity. A total of 35 serotypes of *S. suis* have been described and are defined based on the antigenicity of their capsular polysaccharide (CPS).(6) Evidence accumulated throughout the years has demonstrated a high level of genetic diversity in the *S. suis* species, as shown by sequence analysis of the 16S rRNA and *cpn60* genes: these two methods suggested that serotypes 32 and 34 were divergent, being clustered together at a significant distance from the other serotypes. This led to the suggestion that these two serotypes might be reclassified as a different species (*Streptococcus orisratti*).(27, 28) However, field strains belonging to these serotypes have been isolated from diseased pigs in recent years in Canada and China.(25, 29, 30) More recently, further studies have suggested that the reference strains of additional serotypes (20, 22, 26, and 33) do not belong to the *S. suis* species.(31) As such, there is an urgent need for a consensus in defining whether a serotype belongs to *S. suis* or not.(32)

In order to understand the important roles of the CPS in serotyping and in the interactions of *S. suis* with the host, knowledge regarding the specific structure that confers capsular properties to each individual serotype is necessary. Van Calsteren *et al.* have described the composition and structures of the *S. suis* serotypes 2 and 14 CPS.(33, 34) Still, the chemical composition and structure of the CPS of the serotypes other than 2 and 14 are presently unknown.

3.2 Serological methods

Proper serological typing, which is one of the most important features of the *S. suis* infection diagnosis, must be performed using either a coagglutination test, capillary precipitation test or with Neufeld's capsular reaction using reference antisera.(35, 36) Details on how to perform the coagglutination test have already been published.(37) This test is preferred by many laboratories, especially in North America.(17) Some serotypes cross-react,

indicating the presence of common antigenic determinants. This cross-reaction is probably due to similar or closely related structural features of the CPS. To date, the following cross-reactions have been described: serotype 1/2 with serotypes 1 and 2, serotypes 6 and 16, serotypes 2 and 22, and serotypes 1 and 14.(35, 38) In some cases, absorption is recommended in order to obtain monospecific antisera.(17) However, levels of cross-reactions for some serotypes vary with field strains (M. Gottschalk, 2014, unpubl. observations).

3.3 Serotyping by multiplex PCR

The idea of molecular serotyping by PCR amplification of serotype specific *cps* genes using either a simplex or multiplex PCR is attractive due to the fact that animals are not used for serum production, ease of development and effectiveness. Early studies reported the use of assays targeting some serotypes.(39, 40) Liu *et al*.(41) reported a new protocol using four multiplex PCR assays allowing the detection of the 33 serotypes of *S. suis* (serotypes 1 to 31, 33 and 1/2), but not those related to *S. orisratti* (serotypes 32 and 34). Strains reacting in serology with more than one serotype could be confirmed using this technique. More recently, Okura *et al*. developed a PCR for all 35 described serotypes of *S. suis*.(42) Regardless of the PCR test used, a major disadvantage is the fact that the serotypes 2 and 14 cannot be distinguished from serotypes 1/2 and 1, respectively, since both of these serotype pairs do not possess unique *cps* genes.(43) This represents a major problem for pig isolates since serotypes 1, 1/2, 2, and 14 are commonly isolated in swine.(3, 10) The use of specific antisera is mandatory for such strains. For this reason, only serologically confirmed isolates of serotypes 2 and 14 recovered from pigs were considered in the present review. Although it is also necessary to confirm isolates from humans, it may represent a less important drawback since serotype 1/2 has never been isolated from humans and serotype 1 has been reported in only three non-serologically confirmed cases (at least with both serotypes 1 and 14 antisera), which is important considering these serotypes cross-react (see above).(44, 45) For this reason, isolates from humans reported in the literature as being serotype 2 or 14 based only on PCR reactions (although not serologically confirmed) will be considered as such in the present review.

3.4 "Serotyping" by biochemical identification

In the past, some reported cases of *S. suis* infection in humans have been attributed to serotype 2 based on the biochemical analyses obtained using the rapid multi-test commercial kits mentioned above. While many of these kits claim to differentiate between serotype 1 and 2 strains based on sugar fermentation, there is still no evidence of a correlation between a specific serotype and its biochemical properties.(17, 20, 46) As a result, some human cases have been reported as serotype 2 while others were reported as serotype 1, but since the serotypes of these strains have not been confirmed using antisera (and not even by PCR), their serotypes are herein reported as "unconfirmed by reference antisera or PCR tests".

3.5 Non-typable strains

Some *S. suis* isolates do not agglutinate with any of the typing antisera directed against the 35 serotypes and are identified as non-typable isolates.(29) Non-typable *S. suis* strains may correspond to either truly encapsulated strains that belong to novel, not yet described, encapsulated serotypes, or to non-encapsulated strains, which are impossible to serotype using the serological methods based on CPS antigens. The proportion of non-typable isolates varies between studies depending on the number of serotypes detected using antisera.

Using antisera against all 35 serotypes, Gottschalk *et al*. demonstrated that 89% of non-typable *S. suis* strains presented high surface hydrophobicity, suggesting that they were poorly or non-encapsulated.(25, 47) This was confirmed using transmission electron microscopy, demonstrating that highly hydrophobic strains (74-93%) were non-encapsulated.(25) More than 40% of these non-encapsulated strains have been recently shown to belong to known serotypes using a multiplex PCR for all 35 serotypes.(42) It is also difficult to be certain if these strains were already non-encapsulated when causing disease, or if they lost their CPS during isolation and culture. It has been previously reported that 34% of isolates belonging to serotype 1/2 or 2 recovered from cases of endocarditis in Japan were non-encapsulated due to deletions and insertions in the genes of the CPS locus.(48) It was concluded that although the CPS is considered an important virulence factor for *S. suis*, loss of capsular production might be beneficial to *S. suis* in the course of infective endocarditis. In fact, non-encapsulated strains were shown to possess not only high adhesion properties to

mammalian cells, but also a capacity to form biofilms.(47) Since the sites of isolation of these non-typable strains were similar to those of the most important serotypes (meninges/brain, joints, heart, and lungs), their potential virulence capacity should not be disregarded.

4. Multilocus sequence typing

Being a pathogen capable of causing sporadic cases of infection and epidemics in both pigs and humans, the global surveillance of *S. suis* is very important in order to better understand the epidemiology of this bacterial species.(49) Though different methods based on DNA have been used for the surveillance of *S. suis*, these methods are effective for short-term epidemiology only as they are based on non-characterized genomic differences between isolates.(49) In contrast, MLST distinguishes a large number of genotypes while using genetic variations that accumulate very slowly, in housekeeping genes, and has allowed global and long-term epidemiology for many important meningitis-causing bacteria by determining the STs present within a population.(49, 50)

In 2002, King *et al*. established a model of MLST for *S. suis* using seven different house-keeping genes (*cpn60*, *dpr*, *recA*, *aroA*, *thrA*, *gki*, and *mutS*).(51) Since being established, this MLST model has been used by multiple laboratories throughout the world to determine the STs of *S. suis* strains isolated from pig and human cases of infection. When used alongside serotyping, MLST allows gathering further information about the genetic diversity of the *S. suis* strains within the different serotypes. The popularity of this typing method for *S. suis* continues to increase due to the ease in carrying out the technique (PCR and sequencing) and due to the fact that the data are transferrable and comparable from one laboratory to another. Thanks to the *S. suis* MLST Database, the global distribution of the different *S. suis* STs can be shared and compared. When the MLST data take into consideration the serotypes, it is important that a trustable and complete serotyping system has been applied to the field strains, otherwise the final data are difficult to interpret. More recently, studies have begun combining data obtained from MLST with the presence or absence of different *S. suis* virulence-associated markers at the gene and protein levels including the suilysin (SLY, encoded by the *sly* gene), muramidase-released protein (MRP, encoded by the *mrp* gene),

extracellular factor (EF, encoded by the *epf* gene), and different pili in order to compare STs data with phenotypic characteristics.(52, 53)

5. Methodology used in the present review

In the present review, literature searches were completed using the following databases: Medline (Pubmed; http://www.ncbi.nlm.nih.gov/pubmed/), Web of Science (http://thomsonreuters.com/web-of-science/) and Science Direct (http://www.sciencedirect.com/). Relevant articles in English were used while references in other languages were considered when available. MLST results were obtained from the *S. suis* MLST Database (http://ssuis.mlst.net/). Regarding the distribution of serotypes, the results reported for clinical cases in pigs correspond only to those where serotyping was carried out using antisera and not PCR, while for human cases, reports where serotyping was performed by either antisera or PCR (for serotypes 2 and 14) are both reviewed. This consideration is primarily based on the fact that serotype 1 and serotype 1/2 isolates have not yet been recovered (or confirmed) from human cases of infection. It is important to note that when the correlation between STs and serotypes are reported in this review, it is impossible to confirm the methodology used for the serotyping for many cases.(51) We decided to take the data into consideration, especially for those included in the *S. suis* MLST Database, since the number of cases used for this review would otherwise have been highly limited. For the distribution of STs, the ST complexes represent hyperinvasive lineages to which two or more STs belong as a result of genetic proximity and are based on the MLST phylogeny diagram presented by Lachance *et al.*(54) STs considered to be unrelated to another ST according to this phylogeny diagram are identified as being 'unrelated' to any known ST complex. With the exception of the serotype distribution of *S. suis* human cases, which comprises all cases reported since first described in 1968, distribution of serotypes for clinical cases in pigs and distribution of all STs are based on data published between January 1st, 2002 to December 31st, 2013.

6. Worldwide distribution of serotypes

6.1 Diseased pigs

In order to fully appreciate the prevalence of human infections and the zoonotic potential of *S. suis*, one must understand the situation at the farm level since transmission from diseased pigs or pork-derived products is a prerequisite for infection of humans. The data compiled from studies since January 1st, 2002 are shown in Table 1.

While *S. suis* is part of the normal microflora of pigs and many studies have focused on carriage in healthy pigs, these studies were not included in this review since the serotype distribution in healthy carriers greatly differs from that of clinical strains, with serotype 2 being much less frequently isolated.(55-61). In addition, since *S. suis* is a normal inhabitant of the upper respiratory tract,(6) most of these studies identified only a small part of the real population of each carrier. Finally, in the case where species-specific PCR tests were not used, many of the "untypable" strains detected probably did not belong to the *S. suis* species., The contribution of these commensal strains to the risk posed to humans in close-contact with pigs or with pork-derived products remains to be further studied(14, 62, 63) and seems to occur particularly in immunocompromised patients. The situation may be different when animals are not really "healthy carriers" but rather convalescent animals, carrying in their tonsils virulent strains. However, this situation is almost impossible to define in field studies.

During the last 12 years, more than 4 500 serologically-confirmed strains recovered from diseased pigs have been reported (Table 1). Globally, the predominant *S. suis* serotypes isolated from clinical cases in pigs are, in decreasing order, serotypes 2, 9, 3, 1/2, and 7, along with 15.5% of the strains being non-typable by serotyping. However, there is a clear geographical effect on the distribution of serotypes (see below) and these figures are influenced by the number of published studies.

Almost 70% of studies on worldwide isolates recovered from diseased pigs are from North America (Table 1). Nevertheless, this is not an indication of a higher number of cases but simply of a higher number of published reports and studies. In fact, 97% of North

American data are from Canada and the rest from the United States of America (USA), with no data from Mexico. In North America, serotype 2 is the most prevalent in Canada, while in the USA, it is serotype 3. Meanwhile, the second most prevalent serotype in Canada is serotype 3 and is serotype 2 in the USA. However, only slight differences in percentages have been observed in both countries, demonstrating a similar distribution when the data are combined: both serotypes 2 and 3 are the two most prevalent serotypes isolated from clinical pig cases in North America with 24.3% and 21.0% of prevalence, respectively, followed by serotypes 1/2, 8 and 7. Similar distributions of serotypes in Canada and the USA may be explained by a fluid movement of animals between the two countries.

In South America, only two studies have been published, both from Brazil, which report serotype 2 as being the most prevalent with a mean of 57.6% of cases, followed by, in decreasing order of prevalence, serotypes 1/2, 14, 7, and 9 (Table 1). Interestingly, although no clinical cases in pigs have been published, human cases have been reported in other South American countries (see below).(64-67) On the other hand, no human cases have yet been reported in Brazil.

In Asia, where the vast majority of human cases have been reported (see below), studies on clinical cases in pigs account for only 14.0% of the data available and are provided from China and South Korea only. The most prevalent serotypes in infected pigs are, in decreasing order of prevalence, serotypes 2, 3, 4, 7, and 8 (Table 1). The South Korean study surprisingly reported serotypes 3 and 4 as being predominant, followed by serotype 2 at a low prevalence of only 8.3%. This anomaly may be due to the fact that the paper reported a relatively low number of cases (only 20 isolates) from animals with acute polyserositis only.(68) Even more disturbing is the fact that the information available regarding the status of *S. suis* infections in pigs in Asia is the most scarce of the three continents, while this is the continent where cases of zoonosis occur the most frequently in the general population. Although many hospitals and microbiologists in both Thailand and Vietnam are aware of *S. suis* infections when it comes to diagnosis(2), the four veterinary studies published from these countries investigated healthy or slaughterhouse pigs only.(59, 60, 69, 70) Similarly, there have been 10 human *S. suis* cases reported in Japan between 1994 and 2009, but no complete

study on the distribution of isolates from clinically ill pigs including all serotypes has been recently published. In fact, data from the last epidemiological studies in Japan date back to between 1987 and 1991, more than 20 years ago, which predate the human cases reported in this country and illustrate the necessity to gather data in order to increase our knowledge of the current epidemiological situation.(71) In Hong Kong, where *S. suis* has been reported as the third most common culture-confirmed cause of community-acquired bacterial meningitis, there have been 47 human cases from 1983-2001 and 21 from 2003-2005(12), yet there is a complete absence of epidemiological data regarding *S. suis* in pigs, with the exception of two studies investigating the prevalence of *S. suis* in pork meat sold in wet markets.(72, 73) In Singapore, the Philippines, Laos, and Cambodia, where human cases have occurred during the last decade (Table 2), there are no data available on the epidemiology of *S. suis* infections in pigs.

Important pig producing European countries, such as Denmark, Belgium, France, Germany, Italy, and the United Kingdom have not recently reported the distribution of serotypes recovered from clinical cases in pigs: the last reports from these countries regarded strains isolated between 1990 and 2000.(55, 74) Before the year 2000, serotype 2 was the most common serotype recovered in Italy, France and Spain, whereas serotype 9 was more frequently found in the Netherlands, Germany and Belgium.(76) This lack of information is of high importance. The only two countries with more recent data are Spain and the Netherlands. In fact, in Spain, serotype 2 is no longer the most prevalent serotype, but the second behind serotype 9 and followed by serotypes 7, 8, and 3.(56, 75, 76) In the Netherlands, between 2002 and 2007, serotype 9 was still the most prevalent, followed by serotypes 2, 7, 1, and 4.(77) Although serotype 9 is the most prevalent serotype in Spain and in the Netherlands and was also prevalent in many European countries before the year 2000, no human cases associated with this serotype have yet been reported. Taken together, these two European countries provide a relatively similar serotype distribution with serotypes 9, 2, 7, 8, and 3 in order of importance (Table 1). In studies previous to 2002, serotype 1 also appeared to be prevalent in countries such as Belgium and the United Kingdom(76); however, it is not clear if this is the current situation. The situation is similar in Denmark where only serotype 7 isolates were characterized during the last twelve years and where the last serological study regarded

isolates recovered in 1995-1996.(78, 79) For the time period covered in this review, two reports from France and Italy have been published, although the data from none of these was considered since one of them used serotyping by PCR, detecting serotypes 1 (and 14), 2 (and 1/2), 7, and 9 only and, thus, did not meet the inclusion criterion (see above), while the other studied the distribution of serotypes in healthy animals.(57, 80) Interestingly, human cases have emerged in other European countries in many of which few or no studies on *S. suis* isolation from diseased pigs have been conducted. In Serbia, a recent prevalence study was published using isolates from swab samples taken from dead animals, clinically healthy pigs, slaughterhouse pig carcasses, and butchers' knives, but it was impossible to associate corresponding serotypes to the diseased animals only.(81) In Croatia, only one study pertaining to the antimicrobial susceptibility of *S. suis* type 2 isolates was published,(82) but no epidemiological studies on the distribution of serotypes is available. Moreover, in Greece and Poland, where human cases have been reported, there are still no pig data available. As such, there is an urgent need to evaluate fresh data on the prevalence of *S. suis* in clinical isolates from pigs in Europe where many countries are amongst the more important pig producers in the world.

Finally, there is also a lack of relatively recent serotyping data from Australia and New Zealand. In Australia, where three human cases have been recently reported, the studies concerning serotype distribution in pigs predate these human cases, thus showing that there were once active surveillance studies in this country.(83, 84) One human case of *S. suis* was also reported in New Zealand(85), but no recent studies regarding isolates recovered from diseased pigs have been published in this country.

In summary, there are countries where the serotype distribution of field strains recovered from diseased pigs has been systematically undertaken during the last 12 years, generating data that may influence any analysis of the worldwide distribution of serotypes. There is an urgent need for new data from European countries on the serotype distributions of *S. suis* strains isolated from diseased pigs, especially considering the fact that currently available vaccines are bacterins, which are supposed to confer serotype-specific protection. Some countries with an important pig production, such as Brazil, have few studies on pig

disease and no human cases declared. Other countries commonly report human cases, but data from diseased animals are almost absent.

6.2 Human cases

One of the goals of this review was to compile the serotype distribution of *S. suis* infections in humans, which has not been completed since 1989,(86) and to update the total number of cases from recent reviews on human cases, all of which are excellent comprehensive reviews regarding the clinical features of *S. suis* infections in humans.(3, 18, 87) In the present study, and differently from clinical pig isolates, serotype 2 and 14 isolates were considered to be as such even if identified by PCR, which is unable to differentiate them from serotypes 1/2 and 1, respectively. This consideration is primarily based on the fact that serotype 1 and serotype 1/2 isolates have not yet been recovered (or confirmed) from human cases. A total of 1 642 cases have been reported worldwide as of December 31st, 2013 (Table 2).

Of these cases, serotype 2 was the most frequently reported with 74.7%, followed by serotype 14 with 2.0%. It is important to note that 262 (21.5%) serotype 2 strains and 2 (6.0%) serotype 14 strains were identified by PCR only. The most striking aspect regarding human cases of infection is that 377 cases (23.0%) of all reported cases either do not specify the serotype in the case report or employed a method for serotype identification that was judged inadequate ("biochemical serotyping"). Even though many of these reports claim to be caused by serotype 2, which is probably true, serotyping, or at the very least PCR, should be performed if the strains are still available in order to increase our knowledge of *S. suis* infections in humans. Meanwhile, the remaining five human cases of infection were caused by the following serotypes: 4(14), 5(88), 16(89), 21(67), and 24(88). Since 2011, human *S. suis* cases of infection have been reported for the first time in Cambodia, Chile, French Guiana, Poland, and South Korea.

The vast majority of human cases have occurred in Asia, which account for more than 90% of all reported cases, particularly in Vietnam, Thailand and China. These three countries alone account for 83.6% of all cases worldwide. However, in China, almost all cases were

described during the 1998 and 2005 outbreaks.(16, 90) In East and Southeast Asia, *S. suis* zoonosis should be considered endemic due in part to the high density of pigs, relatively high number of backyard-type production farms, slaughtering practices with the use of few preventive measures, presence of wet markets, and consumption of ill pigs and/or consumption of uncooked or undercooked pork products.(3) In Thailand, most of the reported cases were caused by serotype 2, while serotype 14 is the second highest, the latter accounting for 21 cases of meningitis and sepsis out of a total of 530 cases. However, infections by other serotypes, such as a serotype 5 peritonitis and a serotype 24 sepsis have also been reported. In Vietnam, where *S. suis* is now the most frequent cause of adult bacterial meningitis(10), most cases are also due to serotype 2, six cases of meningitis were caused by serotype 14, and one case of peritonitis by serotype 16. The diversity of infections caused by serotypes other than serotype 2 could be explained by the awareness of diagnosticians to *S. suis* infections. Consequently, this could also explain why Vietnam and Thailand have the largest number of reported cases and a higher probability of encountering atypical cases on a more regular basis. It is also interesting that the patients who suffered infections by serotypes 5, 16 and 24 were also suffering from cirrhosis: a likely explanation is that these infections were the result of immunocompromisation. The compilation of human cases in China includes sporadic cases and the two *S. suis* serotype 2 epidemics, the first in Jiangsu province in 1998, where 14 deaths out of 25 cases occurred(16), and the second in Sichuan province in 2005, where 38 deaths out of 215 cases were reported. This second epidemic still remains the largest outbreak of *S. suis* in humans.(90) These epidemics were unprecedented for *S. suis* infections in humans considering the high incidence of systemic disease, the low number of cases of meningitis, and the high rate of mortality observed.(1) The patients presented cases consisting of either sepsis, meningitis, or STSLS, based on the presence of the following symptoms: sudden onset of high fever, diarrhea, hypotension, blood spots and petechial, clear erythematous blanching rash, and dysfunction of multiple organs, such as acute respiratory distress syndrome, liver and heart failure, disseminated intravascular coagulation, and acute renal failure.(1) Even though China has the largest pig production in the world and is the site of the 1998 and 2005 outbreaks that attracted much attention from the scientific community, the number of reported cases is considerably lower than in Thailand and Vietnam. One possible explanation is that dishes containing raw pork and blood, very popular in Thailand and Vietnam, are uncommon in

China. In fact, since the last outbreak in 2005, only 4 other sporadic human cases were reported in mainland China.(91, 92)

Human cases were also reported in Cambodia, Hong Kong, Japan, Laos, Singapore, South Korea, and Taiwan. It is of interest to note that in Hong Kong, *S. suis* serotype 2 was reported as the third most frequent cause of adult bacterial meningitis.(12, 93) There is also a human case of infection from the Philippines, although it was not officially reported in this country. In fact, even though the infection was diagnosed as *S. suis* serotype 2 meningitis and treated in the USA, the patient had just returned from a seven month vacation in the Philippines where he had consumed raw pork, making this an Asian case and considered as such in this study.(94)

The second continent where most human infections have been described is Europe, accounting for 8.5% of all reported human cases (Table 2), with approximately 71.4% of all European human cases occurring in countries with a highly developed pig industry: the Netherlands, United Kingdom, France, and Spain. Cases were also reported in Austria, Belgium, Croatia, Denmark, Germany, Greece, Ireland, Italy, Poland, Portugal, Serbia, and Sweden. Surprisingly, no cases have yet been reported in Russia, a country with an increased developing swine production. Human cases of *S. suis* infection were reported for the first time in Europe, with the first ever case in Denmark, in 1968. It is interesting to note that all cases reported before 1983 were from Western Europe.(9, 93) Only two countries consider *S. suis* infections in humans as an industrial disease: the United Kingdom and France since 1983 and 1995, respectively.(18) This recognition led to legislation and regulations which may have contributed to reducing the number of cases in both of these countries, with the last human case in the United Kingdom being reported in 2001. In France, although few cases have been reported since 1995, most were related to wild boar hunters. It is known that wild boars also carry *S. suis*.(95) Consequently, hunters should be aware of the necessary precautions when preparing the carcasses, which present the greatest risk.(96, 97) Most human cases of infection contracted from wild boars were serotype 2, with two cases of meningitis caused by serotypes 4 and 14 in the Netherlands, one case of meningitis by serotype 14 in Denmark, and one case of septicemia by serotype 14 in the United Kingdom.(14, 98, 99)

Although being the continent with the highest number of *S. suis* infection reports from diseased pigs (Table 1), only a few sporadic cases of *S. suis* infection in humans have been reported in North America. As previously mentioned, the isolation rate of *S. suis* serotype 2 from diseased pigs is considerable lower than that of Europe or Asia. The lower number of pig cases of disease due to this serotype may be a reason for the lower number of human cases of infection. It has been suggested that *S. suis* strains of serotype 2 from Canada and the USA present lower virulence properties than those from Europe and Asia.(54) For these two countries, confirmed human cases were all serotype 2, with the exception of one serotype 14 case of meningitis in Canada.(100) In the USA, two of the cases were from the continent while the third was from Hawaii, an island in the Pacific Ocean importing pigs from Asia. While the case in Hawaii is considered American (unlike the case from the Philippines), it would perhaps be more appropriate to compare the features of this infection with those from Asia. In fact, the strain recovered from Hawaii presents phenotypic characteristics typical of Asian strains (M. Gottschalk, 2014, unpubl. data).

Sporadic cases were also reported elsewhere throughout the world, such as those in South America (Argentina, Chile and French Guiana), and Oceania (Australia and New Zealand), but when combined, they only account for 0.8% of all reported cases. As was shown by Wertheim *et al*.,(3) sporadic cases of *S. suis* infections in humans are encountered in most regions where pig rearing is important, with notable exceptions being Mexico and Brazil. In the case of Mexico, even though one study evaluated upper respiratory tract colonization of slaughterhouse workers(101) (see below), no official reports on clinical pig cases or human infections are available, which is alarming, knowing full well that in previous studies, clinical strains of *S. suis* isolated from pig and human cases were included.(74, 102) Despite the lack of available reports, this infection is very common in Mexican farms (M. Gottschalk, 2014, unpubl. information). As such, action is required in order to remedy the troubling situation in this country which has well-developed veterinary diagnostic laboratories. It remains to be seen if the absence of human cases is due to a lack of awareness of *S. suis* as a zoonotic pathogen in these regions when diagnosing bacterial infections, or if these strains are of lower virulence, as with some strains from Canada and the USA.

Table 3 shows the frequency of the clinical manifestations of infection by confirmed serotype, illustrating that the serotypes 2 and 14 are involved in similar proportions in meningitis (50-70%) and septicemia (20-25%). Septic shock was defined as a category comprising sepsis, septicemia, bacteremia, and/or STSLS, while meningitis comprises all meningeal-related symptoms. Serotype 2 infections also represented 2.9% of the cases where other afflictions where diagnosed such as endocarditis, septic arthritis, pneumonia, peritonitis, pulmonary edema, myocarditis and other infections. Serotypes 4 and 21 were isolated from cases of meningitis, serotypes 5 and 16 from cases of peritonitis, and serotype 24 from a case of sepsis.

Why reported strains isolated from human cases are almost exclusively serotypes 2 and 14 is puzzling. While the serotypes 3, 9 and 1/2 are amongst the most highly prevalent in diseased pigs after serotype 2, at least in North America, no human infections due to these serotypes have yet been reported in any country. These differences in virulence between pig and human isolates may be partially due to serotype-specific CPS structural features, for which the CPS structures are still unknown, except for serotypes 2 and 14, and/or due to other serotype-specific bacterial factors involved in pathogenesis. We could speculate that the serotypes 2 and 14 are more virulent than the other serotypes based on the observation that four out of the five cases caused by other serotypes (5, 16 and 24) occurred in patients suffering from cirrhosis, which in turn may have immunocompromised them and made them susceptible to serotypes uncommon in human infections.(88, 89) These differences could also be explained by varying serotype-specific colonization abilities. However, many cases caused by serotypes 2 and 14 have also been described in individuals with predisposing conditions. More research is required in order to understand how differences in virulence of *S. suis* strains occurs and if these differences can be associated with specific serotypes.

6.3 Sub-clinically infected humans: Zoonotic potential to workers?

Sub-clinically infected pigs are carriers of *S. suis* mainly in the tonsils.(6) However, this information is not clearly available for humans. Only a handful of studies have

investigated *S. suis* subclinical infection in humans as displayed in Table 4, either by serology (antibodies) or by bacterial detection.

Three of these were serological studies, with two using an indirect ELISA with *S. suis* serotype 2 whole bacteria (formalin-inactivated or not) as antigen (Table 4). In New Zealand, the authors examined sera from 70 pig farmers, 96 dairy farmers, 107 meat inspectors and 16 veterinary students.(103) Twenty-one percent of pig farmers, 10% of meat inspectors and 9% of dairy farmers (most of them also raising pigs on their farms) were positive for "anti-*S. suis* serotype 2 antibodies". It is impossible to be certain if these titers were specific for serotype 2 since cross-reacting antibodies with other *S. suis* serotypes and/or with other bacterial species were probably detected. Antibody titers were associated with longer occupational exposure for both pig farming and meat inspection. In the USA, using a similar antigen, sera from 73 pig-exposed and 67 non-pig-exposed adults from Iowa were titrated. Ten percent of pig-exposed workers were found to be positive compared to only one positive individual (1.5%) from the non-pig-exposed group.(104) Once again, the authors concluded that positive titers were associated with either working with pigs or living on a pig farm for more than ten years, and also found that study participants who worked with both finishing and nursery pigs had 8.8 times the odds of having a positive titer when compared to non-exposed participants. The third serological study, from the Netherlands, is interesting since the authors chose to perform Western Blot analysis, with higher specificity, targeting two virulence-related markers from *S. suis* serotype 2 that are widely present among virulent strains in that country, the MRP and EF proteins, in sera from 102 veterinarians and 191 pig farmers.(105) Results showed that 6% of veterinarians were positive for anti-MRP and 2% were positive for anti-EF, while 1% of pig farmers were positive for anti-MRP and 0.5% were positive for anti-EF. It is important to note that these antigens have not been reported to be present in bacterial species other than *S. suis*. These three serological studies, while the titers obtained cannot be directly compared, illustrate the zoonotic potential of *S. suis* based on the presence of antibodies possibly generated through long-term exposure and/or exposure-related subclinical infections. It should be noted that the presence of antibodies does not guarantee a carriage status for positive individuals.

In addition to these serological studies, three studies have directly focused on carriage in the upper respiratory tract of humans by trying to isolate *S. suis* from pharyngeal or tonsil swabs of pig-exposed workers. (Table 4) In Italy, 10 volunteers from slaughterhouse were swabbed and two were found to be positive for *S. suis* serotype 2.(106) In Mexico, the tonsils from 69 slaughterhouse workers were swabbed and 4 were found to be positive.(101) Of the four strains isolated, one was serotype 2, another was serotype 27, and two were non-typable by serotyping. The virulence of these four strains was also evaluated in mice and it was determined that three of them were mildly virulent (30 to 80% mortality) and one of the non-typable strains was highly virulent (70-90% mortality). Finally, in Germany, the tonsils from 132 meat workers (from either a slaughterhouse or meat processing factory) and 140 controls were swabbed and seven workers were found to be positive carriers of *S. suis* serotype 2 while none of the controls showed positive results. For the seven workers found to be positive carriers, four were retested three weeks later and were confirmed to still be positive carriers, indicating that the bacteria probably remained in the tonsils of healthy individuals for a relatively long period of time, which was also shown to be the case in pigs.(6) However, since these individuals were continuously exposed to pork products, repeated recolonization could not be ruled out.(2) All of the positive workers were employed for an average of 9.9 years ranging from three to 21 years. These three studies regarding the nasopharyngeal carriage all report positive samples in pig-related meat workers and none in the population without occupational exposure to pig. It is also very important to note that of the 13 strains isolated from healthy workers, 84.6% were serotype 2.

Taken together, these studies illustrate the zoonotic potential through long term exposure, either by demonstrating the presence of antibodies towards *S. suis* or by isolating *S. suis* from tonsils or pharynx in pig-exposed workers. It must be noted that the positive rates in the studies of carriers at the upper respiratory tract are probably underestimated since detection of the pathogen was achieved using isolation methods, which are considered as having low sensitivity. Studies using molecular techniques (species-specific PCR) would probably present a significantly higher sensitivity. Exposure to *S. suis* may lead to subclinical infections, induction of antibodies, serious disease, as was the case in the human cases discussed previously, or just an insignificant and transitory atypical colonization of the

mucosal membranes within the respiratory route.(107, 108) The annual risk of developing meningitis due to *S. suis* in the Netherlands was estimated to be 3.5/100 000 for slaughterhouse workers, 2.7/100 000 for pig breeders, and 1.2/100 000 for butchers.(14) Compared to the non-exposed general population (0.002/100 000), the risk for slaughterhouse workers is 1 500 times higher. In the United Kingdom, the risk for butchers is even higher.(109) The annual incidence for the occupational group (direct contact with pigs) in Hong Kong was 32/100 000, 350 times higher than that of the general population (0.09/100 000) and 30 times higher than the homologous group in the Netherlands.(73) In addition, a less dramatic difference between the occupational group and the general population was observed in Hong Kong (350 times higher in Hong Kong versus 1 500 times in The Netherlands). All these differences may be due, at least in part, to a closer contact between people and pork carcasses in Asia.(2) Also, a case-control study conducted in Vietnam from 2006 to 2009 showed that eating high risk dishes common in Vietnam, such as fresh (Tiết Canh) or undercooked blood, tonsils, tongue, stomach, intestines, and uterus from pigs within two weeks prior to admission was the most significant risk factor for *S. suis* infection, followed by exposure to pigs, pork products and preparation of pork in the presence of skin lesions within two weeks prior to admission.(62) The authors also investigated the presence of *S. suis* in pork by-products collected from slaughterhouses and wet/retail markets in Vietnam, and found an 11% contamination rate with *S. suis* serotype 2 in internal organ samples. Unfortunately, they were unable to demonstrate *S. suis* carriage rates in 1 022 healthy individuals or patients without *S. suis* infection using a PCR targeting *S. suis* serotype 2 and 1/2. A PCR for the detection of *S. suis* may have been more sensitive and could possibly have identified potential carriers of other serotypes.(101) The authors suggested that their results, which include 6 positive rectal swabs from patients, strengthen the hypothesis that the gastrointestinal tract may be a route of entry for *S. suis*, which is in agreement with cases following the ingestion of raw pork meat. Similarly, data from Thailand suggest a high incidence rate (6.2/100 000) of *S. suis* infection in the general population in 2010, mostly related to consumption of raw pork products.(110)

7. Worldwide distribution of sequence types

7.1 Global distribution of sequence types

As presented in Fig. 1, different serotype 2 STs predominate in different regions of the world. The ST1 is mostly associated with disease in both pigs and humans in Europe (though the ST20 is important in the Netherlands), Asia (Cambodia, mainland China, Hong Kong, Japan, Thailand, and Vietnam) and Argentina (Tables 5 and 6). Meanwhile, the ST7, responsible for the 1998 and 2005 epidemics, is mostly endemic to mainland China (Tables 5 and 6). North American cases greatly vary from those in Eurasia, with most strains being either ST25 or ST28, two STs also recovered in Thailand and Japan, respectively (Tables 5 and 6). Finally, the ST101 to 104 are endemic to Thailand and appear to be more and more commonly isolated from human cases, especially the ST104 (Table 6). As such, it can be easily observed that the current distribution of the *S. suis* serotype 2 STs greatly varies throughout the world, though data have only been available for a little over a decade, from only a few countries and mostly only for the serotype 2.

Nevertheless, there is an important issue regarding the serotypes attributed to the different strains typed by MLST. Many of the studies, including most of the strains from the *S. suis* MLST Database, do not specify the method used for serotyping. In other cases, such as the study of King *et al*.(51), the authors did not necessarily confirm the serotypes of the strains used. As explained above, the use of PCR for serotyping of serotypes 2 and 14 strains is an inappropriate method, especially for clinical pig cases, even though cases serotyped using this method were still considered for this part of the review. As such, in the cases where more than one serotype was identified for a single ST, it is possible that the serotypes were misidentified and remain to be confirmed using reference antisera. Hence, it is important to keep in mind these discrepancies when analyzing the distribution of STs, particularly for the clinical pig cases. On the other hand, if confirmed, it would be extremely interesting to study strains of different serotypes sharing the same ST, since capsular switching has not been clearly demonstrated for this pathogen.

7.2 Diseased pigs

In contrast to the serotype distribution in diseased pigs (section 6.1), results where the serotype was identified by either serological methods, PCR or unidentified methods and where the ST was determined using the MLST method described by King *et al.*, were taken into consideration for the worldwide distribution of the STs of clinical cases in pigs.(51)

In North America, the majority of MLST studies conducted on *S. suis* strains isolated from diseased pigs have been serotype 2 (Table 5). It was determined that 44% of North American strains are ST25, 51% ST28 and only 5% are ST1.(52) In Canada, the proportion of ST25 and ST28 is similar with 54% and 46%, respectively, but in the USA, 75% of strains were shown to be ST28 and only 10% ST25, while the remaining 15% are ST1.(52) As with North American *S. suis* strains, the majority of European studies have been conducted using serotype 2 strains (Table 5). Most of these studies have demonstrated that ST1 strains are predominately isolated from diseased pigs in the Netherlands, Spain and the United Kingdom.(51, 77, 111) King *et al*. had already associated the serotype 2 ST1 strains with invasive infections.(51) Nevertheless, many strains of serotype 9 have also been recovered from diseased pigs and typed.(77, 111) In both the Netherlands and Spain, serotype 9 isolates were identified as belonging to the ST16, where they represent 43% of strains in the Netherlands.(77) Unlike some countries in Europe, where the serotype 9 is as important as the serotype 2, most *S. suis* strains isolated from diseased pigs in Asia are serotype 2, representing 90% of cases in mainland China.(112) However, as mentioned above, there are relatively few reports of isolation from diseased pigs in Asia. Of these serotype 2 cases, the predominant STs are ST1, ST7 and ST28 (Table 5). In mainland China, Chen *et al.* demonstrated that 22% of serotype 2 strains are ST1 and 77% are ST7.(112) Meanwhile, the few ST28 strains recovered in that country were mostly associated with cases of pneumonia.(113) Regarding Japan, ST1 and ST28 strains isolated from cases of endocarditis in diseased pigs account for 8% and 76% of serotype 2 strains, respectively.(114)

7.3 Human cases

With 97% of all serotype confirmed human cases of *S. suis* infection due to serotype 2, the determination of the STs responsible for these cases becomes highly important (Table 6).

Globally, ST1 strains have been described as mostly responsible for *S. suis* serotype 2 human cases, particularly in South America, Europe and Asia, but also one case in North America.(10, 77, 80, 115-117) Nevertheless, multiple other STs have been described worldwide, though these appear to be endemic to certain geographical regions. For example, the ST20 is important in the Netherlands and France but not in the rest of Europe.(77) ST7 strains, to which belong the strains responsible for the 1998 and 2005 Chinese epidemics, were isolated from human patients only in mainland China and Hong Kong.(116, 117) Meanwhile, ST25 and ST28 strains have been particularly associated with human cases in North America and Japan, respectively.(53, 116, 118) The situation is particular in Thailand, where ST1 and ST104 strains are predominant, causing mainly meningitis and non-meningitis cases, respectively. (53, 115, 119) A few cases of ST25, ST28, ST101, ST102 and ST103 have also been described(115). Interestingly, ST101 to 104 are so far endemic to Thailand only.

Though *S. suis* serotype 14 infections are less frequent in humans than serotype 2 cases, representing 2% of all the serotype-confirmed cases, the number of human infections caused by this serotype appears to be increasing. The ST105 is prevalent in Southeast Asia, particularly in Vietnam and Thailand. In the latter country, 92% of human serotype 14 cases are caused by this ST (Table 6).(10, 120)

Only three human *S. suis* cases, other than those caused by serotypes 2 and 14, have been typed by MLST (Table 6), and all three were described as newly identified STs. Serotype 5, 16 and 24 human cases of infection were identified as ST181, ST106 and ST221, respectively.(88, 89) Interestingly, no data has yet been published on the possible presence of these three newly identified STs for human isolates in diseased pigs.

7.4 Association between pig and human sequence types

Albeit no study has yet associated STs of strains isolated from human cases with those from diseased pigs in the same geographical region, it would seem reasonable to suggest that this association exists, particularly for the serotype 2, as most human strains appear to originate from contact with either pigs or pork by-products. In North America, where serotype 2 ST25 and ST28 strains are predominately isolated from diseased pigs, it is of no surprise that

human ST25 cases were identified.(52, 116, 121) Interestingly, no cases due to ST28 have been diagnosed in humans. It has been suggested that ST25 strains from pigs are more virulent than their ST28 counterparts,(52) which may explain this situation. Moreover, it is interesting to note that even though ST1 strains account for only 5% of isolates from diseased pigs, a human case caused by an ST1 strain was reported in the USA. (122) It is possible that ST1 strains were imported from pigs from Europe and Asia. Being more virulent than their ST25 and ST28 counterparts, it may be hypothesized that a higher number of cases in pigs due to ST1 strains will appear in the near future in North America. Furthermore, similar STs have been described in some Asian countries for both diseased pigs and humans. For example, in Japan, ST1 and ST28 strains have been isolated from both species, while ST1 and ST7 strains have been identified for human and pig isolates in mainland China.(53, 112, 114, 116, 118, 119, 123) The situation is similar in Europe where serotype 2 ST1 clonal complex strains are predominately isolated from diseased pigs in Spain, Italy, the Netherlands, and the United Kingdom and where most human cases have also been typed as belonging to this complex.(51, 77, 80, 111, 124, 125)

This association between pig and human strains within a geographical region, though not definitive and currently only reflecting the situation for the serotype 2, confirms the results obtained by Chatellier *et al*. whereby using randomly amplified polymorphic DNA, they concluded that strains isolated from pigs and humans could not be genotypically distinguished and were similar.(126)

7.5 Association between sequence types and virulence markers

Presently, the most popular virulence markers used in association with STs are the SLY (*sly*), MRP (*mrp*) and EF (*epf*). It is important to note that although the genotypes of strains belonging to other serotypes have been reported, these factors are mainly associated with serotype 2 strains.

Serotype 2 ST1 strains recovered from both clinical pig and human cases have for the most part been genotyped/phenotyped as *sly*+*mrp*+*epf*+/SLY+MRP+EF+, regardless of the geographic origin (whether it be mainland China, Japan, North America or Spain for

both diseased pigs and human cases), which is identical to the serotype 2 ST7 strains isolated from diseased pigs and humans in mainland China.(52, 111, 113, 114, 116, 127) Nevertheless, other important genetic differences vary between ST1 and ST7 strains including the presence of a 89K pathogenicity island in ST7 strains.(128) These ST1 complex strains interestingly differ from not only the human serotype 2 ST104 strains of Thailand which are *sly*+*mrp*-*epf*- but also from the human serotype 2 ST20 strains recovered in the Netherlands that were *epf*-. (77, 115, 116) Also of interest is a human case from Spain where the serotype 2 strain isolated was typed as being a ST3, and presented a large variant of the *mrp*, identified as *mrp** (which has a higher molecular weight), though being *sly*+*epf*+.(125, 129) In Europe, it was determined that strains isolated from diseased pigs belonging to the ST16 complex differ from the ST1 complex strains in being *mrp** rather than *mrp*, while in Spain, the endemic ST123 and ST125 are both *mrp*- and *epf*-.(111) As for ST25 strains isolated from diseased pigs in North America, these were identified as being SLY-MRP-EF-, while the ST28 isolated from North America, mainland China and Japan were *sly*-*mrp*+*epf*- or SLY-MRP+EF-.(52, 113, 114) Though currently not as widely used as the above mentioned virulence markers, different pili (*srtB*, *srtC*, *srtD*, *srtF*, and *srtG*) have also been associated with different STs. It was identified that ST1 strains isolated from both diseased pigs and human cases of serotype 2 infections in Japan and Thailand were *srtBCD*+ and *srtF*+ but *srtG*-.(53) Meanwhile North American ST25 strains isolated from diseased pigs and human cases were *srtF*- and *srtG*+ and ST28 strains isolated from diseased pigs and human cases from North America and Japan were *srtF*+ and *srtG*+. (52, 114)

It still remains difficult to be certain of the association between STs and virulence markers as being universal, but it appears to be representative of populations within a region and may be a useful diagnostic tool with methods identifying the genotypic or phenotypic presence or absence of the different virulence markers.

8. Concluding remarks

S. suis serotype 2 still remains the most isolated serotype and the one most associated with disease in both pigs and humans in most parts of the world. Due to its endemic status in Southeast Asia and the two epidemics it caused in Jiangsu (1998) and Sichuan (2005), China, characterized by STSLS and high mortality rates, this bacterium should no longer be considered merely an important pig pathogen, but also an important and emerging zoonotic agent. Much progress still remains regarding the definition of a "true" *S. suis* serotype and the adoption and use of a complete serotyping system. In this regard, the recent availability of new complete PCR serotyping systems will allow any laboratory in the world to serotype *S. suis* isolates without the need of specific antibodies. The identification of this pathogen by medical and veterinary laboratories significantly varies among countries, and in only a few regions both are well developed. In some important pig producing countries in the Americas, such as Canada, the USA, Mexico, and Brazil, diagnostic laboratories in human medicine should be more aware of the zoonotic potential of *S. suis* due to occupational exposure to pigs and/or pork-derived products. The scientific community should request that the specific serotype of each reported case in humans be identified using acceptable techniques to keep useful epidemiological data. On the other hand, in some countries in Asia where human cases are routinely reported, the number of studies provided by diagnostic laboratories working in veterinary medicine should be significantly increased, since there are almost no data regarding strains recovered from diseased animals. The same applies to European countries which are important pig producers and from which no data regarding the distribution of serotypes from diseased animals were reported in the last twelve years, hampering the use of species-specific protective bacterins.

Though the MLST data currently available for *S. suis* strains isolated from both diseased pigs and human cases of infection come from different countries throughout the world, there still remains a long way to go before a complete picture of the current situation of *S. suis* can be obtained. Yet, this picture is necessary as it could hint at both dominating and emerging STs and could possibly help identify epidemic strains quickly and to categorize the zoonotic potential of specific STs. With the dissemination of information about STs across the

internet, particularly thanks to the *S. suis* MLST Database, it would be important to set up a complete and reliable serotype identification of the different strains added. Albeit this database is a great tool that facilitates the sharing of knowledge, the lack of many details pertaining to each strain such as the serotype, method used for serotyping, health status of the host, and host itself, limit the full potential of the STs as was demonstrated in this review where many strains could not be included. Furthermore, in the absence of identification of the serotyping method, it is impossible to confirm if the serotype indicated is correct. Nevertheless, the global distribution of *S. suis*, particularly serotype 2 strains greatly varies, an aspect that could be useful in developing diagnostic and preventive tools specific for a particular geographical distribution. Despite the fact that studies have recently associated virulence markers and profiles with given STs, the biological significance of these associations still needs to be studied, as only a handful of studies have compared STs from different geographical origins. In fact, some STs found in North America and described as low virulent are frequently isolated from patients in Asia. There is an urgent need to compare virulence properties of strains of similar STs from different geographical origins. Finally, though still tentative, the possibility of associating strains from diseased pigs and human cases in a given region, as determined based on serotypes and STs, would provide further epidemiological support for the zoonotic potential of this pig pathogen, demonstrating the need for proper hygiene practises in order to reduce the risk of zoonotic infections. With more and more laboratories using a complete serotyping system and MLST as a tool for *S. suis* classification, we will one day have the complete picture regarding the *S. suis* distribution.

Acknowledgements

The research carried out on *Streptococcus suis* in our laboratories is funded by the Natural Sciences and Engineering Research Council of Canada [grant #154280 to MG and 342150-07 to MS] and by China-Canada Joint Health Research Initiative financed by Canadian Institutes of Health Research and the National Natural Science Foundation of China (812111251). This publication made use of the Multilocus Sequence Typing Website (http://www.mlst.net) at Imperial College London developed by David Aanensen and funded by the Welcome Trust.

References

1. Gottschalk M, Segura M, Xu J. *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. Anim Health Res Rev. 2007;8(1):29-45.
2. Gottschalk M, Xu J, Lecours M-P, Grenier D, Fittipaldi N, Segura M. *Streptococcus suis* Infections in Humans: What is the prognosis for Western countries? (Part II). Clin Microbiol Newsl. 2010;32(13):97-102.
3. Wertheim HF, Nghia HD, Taylor W, Schultsz C. *Streptococcus suis*: an emerging human pathogen. Clin Infect Dis. 2009;48(5):617-25.
4. Fittipaldi N, Segura M, Grenier D, Gottschalk M. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent *Streptococcus suis*. Future Microbiol. 2012;7(2):259-79.
5. Baums CG, Valentin-Weigand P. Surface-associated and secreted factors of *Streptococcus suis* in epidemiology, pathogenesis and vaccine development. Anim Health Res Rev. 2009;10(01):65-83.
6. Higgins R, Gottschalk M. Streptococcal diseases. In: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ, editors. Diseases of swine. 9th ed. Ames, USA: Blackwell Publishing; 2006. p. 769-83.
7. Sanford SE, Tilker ME. *Streptococcus suis* type II-associated diseases in swine: observations of a one-year study. J Am Vet Med Assoc. 1982;181(7):673-6.
8. Staats JJ, Feder I, Okwumabua O, Chengappa MM. *Streptococcus suis*: past and present. Vet Res Commun. 1997;21(6):381-407.
9. Perch B, Kristjansen P, Skadhauge K. Group R streptococci pathogenic for man. Two cases of meningitis and one fatal case of sepsis. Acta Pathol Microbiol Scand. 1968;74(1):69-76.
10. Mai NT, Hoa NT, Nga TV, Linh le D, Chau TT, Sinh DX, et al. *Streptococcus suis* meningitis in adults in Vietnam. Clin Infect Dis. 2008;46(5):659-67.
11. Suankratay C, Intalapaporn P, Nunthapisud P, Arunyingmongkol K, Wilde H. *Streptococcus suis* meningitis in Thailand. Southeast Asian J Trop Med Public Health. 2004;35(4):868-76.
12. Hui AC, Ng KC, Tong PY, Mok V, Chow KM, Wu A, et al. Bacterial meningitis in Hong Kong: 10-years' experience. Clin Neurol Neurosurg. 2005;107(5):366-70.
13. Fongcom A, Pruksakorn S, Mongkol R, Tharavichitkul P, Yoonim N. *Streptococcus suis* infection in northern Thailand. J Med Assoc Thai. 2001;84(10):1502-8.
14. Arends JP, Zanen HC. Meningitis caused by *Streptococcus suis* in humans. Rev Infect Dis. 1988;10(1):131-7.
15. Huang YT, Teng LJ, Ho SW, Hsueh PR. *Streptococcus suis* infection. J Microbiol Immunol Infect. 2005;38(5):306-13.
16. Tang J, Wang C, Feng Y, Yang W, Song H, Chen Z, et al. Streptococcal toxic shock syndrome caused by *Streptococcus suis* serotype 2. PLoS Med. 2006;3(5):e151.
17. Higgins R, Gottschalk M. An update on *Streptococcus suis* identification. J Vet Diagn Invest. 1990;2(3):249-52.
18. Gottschalk M, Xu J, Calzas C, Segura M. *Streptococcus suis*: a new emerging or an old neglected zoonotic pathogen? Future Microbiol. 2010;5(3):371-91.
19. Lutticken R, Temme N, Hahn G, Bartelheimer EW. Meningitis caused by *Streptococcus suis*: case report and review of the literature. Infection. 1986;14(4):181-5.

20. Michaud S, Duperval R, Higgins R. *Streptococcus suis* meningitis: First case reported in Quebec. Can J Infect Dis. 1996;7(5):329-31.
21. Tsai HC, Lee SS, Wann SR, Huang TS, Chen YS, Liu YC. *Streptococcus suis* meningitis with ventriculoperitoneal shunt infection and spondylodiscitis. J Formos Med Assoc. 2005;104(12):948-50.
22. Yen MY, Liu YC, Wang JH, Chen YS, Wang YH, Cheng DL. *Streptococcus suis* meningitis complicated with permanent perceptive deafness: report of a case. J Formos Med Assoc. 1994;93(4):349-51.
23. Donsakul K, Dejthevaporn C, Witoonpanich R. *Streptococcus suis* infection: clinical features and diagnostic pitfalls. Southeast Asian J Trop Med Public Health. 2003;34(1):154-8.
24. Okwumabua O, O'Connor M, Shull E. A polymerase chain reaction (PCR) assay specific for *Streptococcus suis* based on the gene encoding the glutamate dehydrogenase. FEMS Microbiol Lett. 2003;218(1):79-84.
25. Gottschalk M, Lacouture S, Bonifait L, Roy D, Fittipaldi N, Grenier D. Characterization of *Streptococcus suis* isolates recovered between 2008 and 2011 from diseased pigs in Quebec, Canada. Vet Microbiol. 2013;162(2-4):819-25.
26. Tien le HT, Sugiyama N, Duangsonk K, Tharavichitkul P, Osawa R. Phenotypic and PCR-based identification of bacterial strains isolated from patients with suspected *Streptococcus suis* infection in northern Thailand. Jpn J Infect Dis. 2012;65(2):171-4.
27. Hill JE, Gottschalk M, Brousseau R, Harel J, Hemmingsen SM, Goh SH. Biochemical analysis, cpn60 and 16S rDNA sequence data indicate that *Streptococcus suis* serotypes 32 and 34, isolated from pigs, are *Streptococcus orisratti*. Vet Microbiol. 2005;107(1–2):63-9.
28. Brousseau R, Hill JE, Prefontaine G, Goh SH, Harel J, Hemmingsen SM. *Streptococcus suis* serotypes characterized by analysis of chaperonin 60 gene sequences. Appl Environ Microbiol. 2001;67(10):4828-33.
29. Messier S, Lacouture S, Gottschalk M. Distribution of *Streptococcus suis* capsular types from 2001 to 2007. Can Vet J. 2008;49(5):461-2.
30. Wang K, Sun X, Lu C. Development of rapid serotype-specific PCR assays for eight serotypes of *Streptococcus suis*. J Clin Microbiol. 2012;50(10):3329-34.
31. Tien le HT, Nishibori T, Nishitani Y, Nomoto R, Osawa R. Reappraisal of the taxonomy of *Streptococcus suis* serotypes 20, 22, 26, and 33 based on DNA-DNA homology and sodA and recN phylogenies. Vet Microbiol. 2013;162(2-4):842-9.
32. Segura M, Zheng H, Greeff A, Gao GF, Grenier D, Jiang Y, et al. Latest developments on *Streptococcus suis*: an emerging zoonotic pathogen: part 1. Future Microbiol. 2014;9:441-4.
33. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. Biochem Cell Biol. 2010;88(3):513-25.
34. Van Calsteren MR, Gagnon F, Calzas C, Goyette-Desjardins G, Okura M, Takamatsu D, et al. Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide. Biochem Cell Biol. 2013;91(2):49-58.
35. Gottschalk M, Higgins R, Jacques M, Mittal KR, Henrichsen J. Description of 14 new capsular types of *Streptococcus suis*. J Clin Microbiol. 1989;27(12):2633-6.

36. Gottschalk M, Higgins R, Jacques M, Beaudoin M, Henrichsen J. Isolation and characterization of *Streptococcus suis* capsular types 9-22. J Vet Diagn Invest. 1991;3(1):60-5.
37. Gottschalk M, Higgins R, Boudreau M. Use of polyvalent coagglutination reagents for serotyping of *Streptococcus suis*. J Clin Microbiol. 1993;31(8):2192-4.
38. Perch B, Kjems E, Slot P, Pedersen KB. Biochemical and serological properties of R, S, and RS streptococci. Acta Pathol Microbiol Scand B. 1981;89(3):167-71.
39. Wisselink HJ, Joosten JJ, Smith HE. Multiplex PCR assays for simultaneous detection of six major serotypes and two virulence-associated phenotypes of *Streptococcus suis* in tonsillar specimens from pigs. J Clin Microbiol. 2002;40(8):2922-9.
40. Marois C, Bougeard S, Gottschalk M, Kobisch M. Multiplex PCR assay for detection of *Streptococcus suis* species and serotypes 2 and 1/2 in tonsils of live and dead pigs. J Clin Microbiol. 2004;42(7):3169-75.
41. Liu Z, Zheng H, Gottschalk M, Bai X, Lan R, Ji S, et al. Development of multiplex PCR assays for the identification of the 33 serotypes of *Streptococcus suis*. PLoS One. 2013;8(8):e72070.
42. Okura M, Lachance C, Osaki M, Sekizaki T, Maruyama F, Nozawa T, et al. Development of a Two-Step Multiplex PCR Assay for Typing of Capsular Polysaccharide Synthesis Gene Clusters of Streptococcus suis. J Clin Microbiol. 2014;52(5):1714-9.
43. Okura M, Takamatsu D, Maruyama F, Nozawa T, Nakagawa I, Osaki M, et al. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: potential mechanisms for generation of capsular variation. Appl Environ Microbiol. 2013;79(8):2796-806.
44. Kopic J, Paradzik MT, Pandak N. *Streptococcus suis* infection as a cause of severe illness: 2 cases from Croatia. Scand J Infect Dis. 2002;34(9):683-4.
45. Vilaichone RK, Vilaichone W, Nunthapisud P, Wilde H. *Streptococcus suis* infection in Thailand. J Med Assoc Thai. 2002;85:S109-17.
46. Prieto C, Garcia FJ, Suarez P, Imaz M, Castro JM. Biochemical traits and antimicrobial susceptibility of *Streptococcus suis* isolated from slaughtered pigs. Zentralbl Veterinarmed B. 1994;41(9):608-17.
47. Bonifait L, Gottschalk M, Grenier D. Cell surface characteristics of nontypeable isolates of *Streptococcus suis*. FEMS Microbiol Lett. 2010;311(2):160-6.
48. Lakkitjaroen N, Takamatsu D, Okura M, Sato M, Osaki M, Sekizaki T. Loss of capsule among *Streptococcus suis* isolates from porcine endocarditis and its biological significance. J Med Microbiol. 2011;60(Pt 11):1669-76.
49. Brehony C, Jolley KA, Maiden MC. Multilocus sequence typing for global surveillance of meningococcal disease. FEMS Microbiol Rev. 2007;31(1):15-26.
50. Maiden MC. Multilocus sequence typing of bacteria. Annu Rev Microbiol. 2006;60:561-88.
51. King SJ, Leigh JA, Heath PJ, Luque I, Tarradas C, Dowson CG, et al. Development of a multilocus sequence typing scheme for the pig pathogen *Streptococcus suis*: identification of virulent clones and potential capsular serotype exchange. J Clin Microbiol. 2002;40(10):3671-80.

52. Fittipaldi N, Xu J, Lacouture S, Tharavichitkul P, Osaki M, Sekizaki T, et al. Lineage and virulence of *Streptococcus suis* serotype 2 isolates from North America. Emerg Infect Dis. 2011;17(12):2239-44.
53. Takamatsu D, Nishino H, Ishiji T, Ishii J, Osaki M, Fittipaldi N, et al. Genetic organization and preferential distribution of putative pilus gene clusters in *Streptococcus suis*. Vet Microbiol. 2009;138(1-2):132-9.
54. Lachance C, Gottschalk M, Gerber PP, Lemire P, Xu J, Segura M. Exacerbated type II interferon response drives hypervirulence and toxic shock by an emergent epidemic strain of *Streptococcus suis*. Infect Immun. 2013;81(6):1928-39.
55. Wisselink HJ, Smith HE, Stockhofe-Zurwieden N, Peperkamp K, Vecht U. Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of *Streptococcus suis* strains isolated from diseased pigs in seven European countries. Vet Microbiol. 2000;74(3):237-48.
56. Luque I, Blume V, Borge C, Vela AI, Perea JA, Marquez JM, et al. Genetic analysis of *Streptococcus suis* isolates recovered from diseased and healthy carrier pigs at different stages of production on a pig farm. Vet J. 2010;186(3):396-8.
57. Marois C, Le Devendec L, Gottschalk M, Kobisch M. Detection and molecular typing of *Streptococcus suis* in tonsils from live pigs in France. Can J Vet Res. 2007;71(1):14-22.
58. Wang K, Zhang W, Li X, Lu C, Chen J, Fan W, et al. Characterization of *Streptococcus suis* isolates from slaughter swine. Curr Microbiol. 2013;66(4):344-9.
59. Ngo TH, Tran TB, Tran TT, Nguyen VD, Campbell J, Pham HA, et al. Slaughterhouse pigs are a major reservoir of *Streptococcus suis* serotype 2 capable of causing human infection in southern Vietnam. PLoS One. 2011;6(3):e17943.
60. Kerdsin A, Dejsirilert S, Akeda Y, Sekizaki T, Hamada S, Gottschalk M, et al. Fifteen *Streptococcus suis* serotypes identified by multiplex PCR. J Med Microbiol. 2012;61(Pt 12):1669-72.
61. Zhang CP, Ning YB, Zhang ZQ, Song L, Qiu HS, Gao HY, et al. [Distributions of pathogenic capsular types and in vitro antimicrobial susceptibility of different serotypes of *Streptococcus suis* isolated from clinically healthy sows from 10 provinces in China]. Zhonghua liu xing bing xue za zhi = Zhonghua liuxingbingxue zazhi. 2009;30(3):235-8.
62. Nghia HD, Tu le TP, Wolbers M, Thai CQ, Hoang NV, Nga TV, et al. Risk factors of *Streptococcus suis* infection in Vietnam. A case-control study. PLoS One. 2011;6(3):e17604.
63. Vilaichone RK, Mahachai V, Nunthapisud P. *Streptococcus suis* peritonitis: case report. J Med Assoc Thai. 2000;83(10):1274-7.
64. Lopreto C, Lopardo HA, Bardi MC, Gottschalk M. [Primary *Streptococcus suis* meningitis: first case in humans described in Latin America]. Enferm Infecc Microbiol Clin. 2005;23(2):110.
65. Koch E, Fuentes G, Carvajal R, Palma R, Aguirre V, Cruz C, et al. [*Streptococcus suis* meningitis in pig farmers: report of first two cases in Chile]. Rev Chilena Infectol. 2013;30(5):557-61.
66. Alarcon P, Araya P, Aguayo C, Fernandez J, Illesca V, Zaror A, et al. [Laboratory confirmation of *Streptococcus suis* in Chile]. Rev Chilena Infectol. 2013;30(5):539-40.
67. Callejo R, Prieto M, Salamone F, Auger J, Goyette-Desjardins G, Gottschalk M. Atypical *Streptococcus suis* in man, Argentina, 2013. Emerg Infect Dis. 2014;20(3):500-2.

68. Kim D, Han K, Oh Y, Kim CH, Kang I, Lee J, et al. Distribution of capsular serotypes and virulence markers of *Streptococcus suis* isolated from pigs with polyserositis in Korea. Can J Vet Res. 2010;74(4):314-6.
69. Padungtod P, Tharavichitkul P, Junya S, Chaisowong W, Kadohira M, Makino S, et al. Incidence and presence of virulence factors of *Streptococcus suis* infection in slaughtered pigs from Chiang Mai, Thailand. Southeast Asian J Trop Med Public Health. 2010;41(6):1454-61.
70. Hoa NT, Chieu TT, Do Dung S, Long NT, Hieu TQ, Luc NT, et al. *Streptococcus suis* and porcine reproductive and respiratory syndrome, Vietnam. Emerg Infect Dis. 2013;19(2):331-3.
71. Kataoka Y, Sugimoto C, Nakazawa M, Morozumi T, Kashiwazaki M. The epidemiological studies of *Streptococcus suis* infections in Japan from 1987 to 1991. J Vet Med Sci. 1993;55(4):623-6.
72. Ip M, Fung KS, Chi F, Cheuk ES, Chau SS, Wong BW, et al. *Streptococcus suis* in Hong Kong. Diagn Microbiol Infect Dis. 2007;57(1):15-20.
73. Ma E, Chung PH, So T, Wong L, Choi KM, Cheung DT, et al. *Streptococcus suis* infection in Hong Kong: an emerging infectious disease? Epidemiol Infect. 2008;136(12):1691-7.
74. Marie J, Morvan H, Berthelot-Herault F, Sanders P, Kempf I, Gautier-Bouchardon AV, et al. Antimicrobial susceptibility of *Streptococcus suis* isolated from swine in France and from humans in different countries between 1996 and 2000. J Antimicrob Chemother. 2002;50(2):201-9.
75. Vela AI, Goyache J, Tarradas C, Luque I, Mateos A, Moreno MA, et al. Analysis of genetic diversity of *Streptococcus suis* clinical isolates from pigs in Spain by pulsed-field gel electrophoresis. J Clin Microbiol. 2003;41(6):2498-502.
76. Tarradas C, Perea A, Vela AI, Goyache J, Dominguez L, Fernandez-Garaizabal JF, et al. Distribution of serotypes of *Streptococcus suis* isolated from diseased pigs in Spain. Vet Rec. 2004;154(21):665-6.
77. Schultsz C, Jansen E, Keijzers W, Rothkamp A, Duim B, Wagenaar JA, et al. Differences in the population structure of invasive *Streptococcus suis* strains isolated from pigs and from humans in The Netherlands. PLoS One. 2012;7(5):e33854.
78. Tian Y, Aarestrup FM, Lu CP. Characterization of *Streptococcus suis* serotype 7 isolates from diseased pigs in Denmark. Vet Microbiol. 2004;103(1-2):55-62.
79. Aarestrup FM, Jorsal SE, Jensen NE. Serological characterization and antimicrobial susceptibility of *Streptococcus suis* isolates from diagnostic samples in Denmark during 1995 and 1996. Vet Microbiol. 1998;60(1):59-66.
80. Princivalli MS, Palmieri C, Magi G, Vignaroli C, Manzin A, Camporese A, et al. Genetic diversity of *Streptococcus suis* clinical isolates from pigs and humans in Italy (2003-2007). Euro Surveill. 2009;14(33):pii=19010.
81. Stanojkovic A, Asanin R, Misic D, Asanin J, Stanojkovic-Sebic A. The presence and serological types of *Streptococcus suis* strains isolated from pigs originating from some farms in Serbia. Fresenius Environmental Bulletin. 2012;21(11C):3558-61.
82. Seol B, Kelneric Z, Hajsig D, Madic J, Naglic T. Susceptibility to antimicrobial agents of *Streptococcus suis* capsular type 2 strains isolated from pigs. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg. 1996;283(3):328-31.

83. Buddle JR, Jones JE, Pass DA, Robertson J. The isolation of *Streptococcus suis* type II from a pig with meningitis. Aust Vet J. 1981;57(9):437-8.
84. Mwaniki CG, Robertson ID, Trott DJ, Atyeo RF, Lee BJ, Hampson DJ. Clonal analysis and virulence of Australian isolates of *Streptococcus suis* type 2. Epidemiol Infect. 1994;113(2):321-34.
85. Dickie AS, Bremner DA, Wong PY, North JD, Robertson ID. *Streptococcus suis* bacteraemia. N Z Med J. 1987;100(835):677-8.
86. Kohler W, Queisser H, Kunter E, Sawitzki R, Frach G. [Type 2 *Streptococcus suis* (R-Streptococci) as pathogens of occupational diseases. Report of a case and a review of the literature]. Z Gesamte Inn Med. 1989;44(5):144-8.
87. Lun ZR, Wang QP, Chen XG, Li AX, Zhu XQ. *Streptococcus suis*: an emerging zoonotic pathogen. Lancet Infect Dis. 2007;7(3):201-9.
88. Kerdsin A, Dejsirilert S, Sawanpanyalert P, Boonnark A, Noithachang W, Sriyakum D, et al. Sepsis and spontaneous bacterial peritonitis in Thailand. Lancet. 2011;378(9794):960.
89. Nghia HD, Hoa NT, Linh le D, Campbell J, Diep TS, Chau NV, et al. Human case of *Streptococcus suis* serotype 16 infection. Emerg Infect Dis. 2008;14(1):155-7.
90. Yu H, Jing H, Chen Z, Zheng H, Zhu X, Wang H, et al. Human *Streptococcus suis* outbreak, Sichuan, China. Emerg Infect Dis. 2006;12(6):914-20.
91. Zhu Y, Tan Z, Zhu L, Pan H, Zhang X, Xu L, et al. *Streptococcus suis* serotype 2 caused streptococcal toxic shock syndrome (STSS) in a patient. Journal of Nanjing Medical University. 2008;22(5):313-6.
92. Ng HW. Overwhelming postsplenectomy sepsis presenting as purpura fulminans due to *Streptococcus suis* infection. Hong Kong Journal of Emergency Medicine. 2013;20(3):155-60.
93. Chau PY, Huang CY, Kay R. *Streptococcus suis* meningitis. An important underdiagnosed disease in Hong Kong. Med J Aust. 1983;1(9):414-6, 7.
94. Lee GT, Chiu CY, Haller BL, Denn PM, Hall CS, Gerberding JL. *Streptococcus suis* meningitis, United States. Emerg Infect Dis. 2008;14(1):183-5.
95. Bonmarchand G, Massari P, Humbert G, Leroy J, Morel A, Lemeland JF, et al. Group R streptococci: wild boars as a second reservoir. Scand J Infect Dis. 1985;17(1):121-2.
96. Piech C, Guettrot-Imbert G, Delevaux I, André M, Lhoste A, Aumaître O. [*Streptococcus suis* spondylodiscitis and oligoarthritis in a hunter]. Rev Med Intern. 2009;30(0):S142.
97. Rosenstingl S, Rouede Y, Galanti MJ, Gatfosse M. [*Streptococcus suis* septicaemia in a hunter's wife]. Med Maladies Infect. 2008;38(0):S177.
98. Poggenborg R, Gaini S, Kjaeldgaard P, Christensen JJ. *Streptococcus suis*: meningitis, spondylodiscitis and bacteraemia with a serotype 14 strain. Scand J Infect Dis. 2008;40(4):346-9.
99. Watkins EJ, Brooksby P, Schweiger MS, Enright SM. Septicaemia in a pig-farm worker. Lancet. 2001;357(9249):38.
100. Haleis A, Alfa M, Gottschalk M, Bernard K, Ronald A, Manickam K. Meningitis caused by *Streptococcus suis* serotype 14, North America. Emerg Infect Dis. 2009;15(2):350-2.
101. Rojas MT, Gottschalk M, Velazquez Ordonez V. [Virulence evaluation of serotypes of *Streptococcus suis* isolated in slaughterhouse workers in the valley of Toluca, State of Mexico, Mexico]. Vet Mex. 2001;32(3):201-5.

102. Quessy S, Dubreuil JD, Caya M, Higgins R. Discrimination of virulent and avirulent *Streptococcus suis* capsular type 2 isolates from different geographical origins. Infect Immun. 1995;63(5):1975-9.
103. Robertson ID, Blackmore DK. Occupational exposure to *Streptococcus suis* type 2. Epidemiol Infect. 1989;103(1):157-64.
104. Smith TC, Capuano AW, Boese B, Myers KP, Gray GC. Exposure to *Streptococcus suis* among US swine workers. Emerg Infect Dis. 2008;14(12):1925-7.
105. Elbers AR, Vecht U, Osterhaus AD, Groen J, Wisselink HJ, Diepersloot RJ, et al. Low prevalence of antibodies against the zoonotic agents *Brucella abortus*, *Leptospira* spp., *Streptococcus suis* serotype II, hantavirus, and lymphocytic choriomeningitis virus among veterinarians and pig farmers in the southern part of The Netherlands. Vet Q. 1999;21(2):50-4.
106. Sala V, Colombo A, Gerola L. [Infection Risks of *Streptococcus suis* Type 2 Localizations in Slaughtered Swines]. Arch Vet It. 1989;40(3):180-4.
107. Kay R, Cheng AF, Tse CY. *Streptococcus suis* infection in Hong Kong. QJM. 1995;88(1):39-47.
108. Strangmann E, Froleke H, Kohse KP. Septic shock caused by *Streptococcus suis*: case report and investigation of a risk group. Int J Hyg Environ Health. 2002;205(5):385-92.
109. Walsh B, Williams AE, Satsangi J. *Streptococcus suis* type 2: pathogenesis and clinical disease. Rev Med Microbiol. 1992;3:65-71.
110. Takeuchi D, Kerdsin A, Pienpringam A, Loetthong P, Samerchea S, Luangsuk P, et al. Population-based study of *Streptococcus suis* infection in humans in Phayao Province in northern Thailand. PLoS One. 2012;7(2):e31265.
111. Blume V, Luque I, Vela AI, Borge C, Maldonado A, Dominguez L, et al. Genetic and virulence-phenotype characterization of serotypes 2 and 9 of *Streptococcus suis* swine isolates. Int Microbiol. 2009;12(3):161-6.
112. Chen L, Song Y, Wei Z, He H, Zhang A, Jin M. Antimicrobial susceptibility, tetracycline and erythromycin resistance genes, and multilocus sequence typing of *Streptococcus suis* isolates from diseased pigs in China. J Vet Med Sci. 2013;75(5):583-7.
113. Zhu W, Wu C, Sun X, Zhang A, Zhu J, Hua Y, et al. Characterization of *Streptococcus suis* serotype 2 isolates from China. Vet Microbiol. 2013;166(3–4):527-34.
114. Onishi H, Sugawara M, Okura M, Osaki M, Takamatsu D. Prevalence of *Streptococcus suis* genotypes in isolates from porcine endocarditis in East Japan. J Vet Med Sci. 2012;74(12):1681-4.
115. Kerdsin A, Dejsirilert S, Puangpatra P, Sripakdee S, Chumla K, Boonkerd N, et al. Genotypic profile of *Streptococcus suis* serotype 2 and clinical features of infection in humans, Thailand. Emerg Infect Dis. 2011;17(5):835-42.
116. Li W, Ye C, Jing H, Cui Z, Bai X, Jin D, et al. *Streptococcus suis* outbreak investigation using multiple-locus variable tandem repeat number analysis. Microbiol Immunol. 2010;54(7):380-8.
117. Luey CK, Chu YW, Cheung TK, Law CC, Chu MY, Cheung DT, et al. Rapid pulsed-field gel electrophoresis protocol for subtyping of *Streptococcus suis* serotype 2. J Microbiol Methods. 2007;68(3):648-50.
118. Chang B, Wada A, Ikebe T, Ohnishi M, Mita K, Endo M, et al. Characteristics of *Streptococcus suis* isolated from patients in Japan. Jpn J Infect Dis. 2006;59(6):397-9.

119. Takamatsu D, Wongsawan K, Osaki M, Nishino H, Ishiji T, Tharavichitkul P, et al. *Streptococcus suis* in humans, Thailand. Emerg Infect Dis. 2008;14(1):181-3.
120. Kerdsin A, Oishi K, Sripakdee S, Boonkerd N, Polwichai P, Nakamura S, et al. Clonal dissemination of human isolates of *Streptococcus suis* serotype 14 in Thailand. J Med Microbiol. 2009;58(Pt 11):1508-13.
121. Fowler HN, Brown P, Rovira A, Shade B, Klammer K, Smith K, et al. *Streptococcus suis* meningitis in swine worker, Minnesota, USA. Emerg Infect Dis. 2013;19(2):330-1.
122. Willenburg KS, Sentochnik DE, Zadoks RN. Human *Streptococcus suis* meningitis in the United States. N Engl J Med. 2006;354(12):1325.
123. Ye C, Bai X, Zhang J, Jing H, Zheng H, Du H, et al. Spread of *Streptococcus suis* sequence type 7, China. Emerg Infect Dis. 2008;14(5):787-91.
124. de Greeff A, Wisselink HJ, de Bree FM, Schultsz C, Baums CG, Thi HN, et al. Genetic diversity of *Streptococcus suis* isolates as determined by comparative genome hybridization. BMC Microbiol. 2011;11:161.
125. Vela AI, Aspiroz C, Fortuno B, Tirado G, Sierra J, Martinez R, et al. Meningitis caused by an unusual genotype (ST3) of *Streptococcus suis*. Infection. 2013;41(3):701-3.
126. Chatellier S, Gottschalk M, Higgins R, Brousseau R, Harel J. Relatedness of *Streptococcus suis* serotype 2 isolates from different geographic origins as evaluated by molecular fingerprinting and phenotyping. J Clin Microbiol. 1999;37(2):362-6.
127. Tang Y, Zhao H, Wu W, Wu D, Li X, Fang W. Genetic and virulence characterization of *Streptococcus suis* type 2 isolates from swine in the provinces of Zhejiang and Henan, China. Folia Microbiol (Praha). 2011;56(6):541-8.
128. Chen C, Tang J, Dong W, Wang C, Feng Y, Wang J, et al. A glimpse of streptococcal toxic shock syndrome from comparative genomics of *S. suis* 2 Chinese isolates. PLoS One. 2007;2(3):e315.
129. Vecht U, Wisselink HJ, Jellema ML, Smith HE. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect Immun. 1991;59(9):3156-62.
130. Fittipaldi N, Fuller TE, Teel JF, Wilson TL, Wolfram TJ, Lowery DE, et al. Serotype distribution and production of muramidase-released protein, extracellular factor and suilysin by field strains of *Streptococcus suis* isolated in the United States. Vet Microbiol. 2009;139(3-4):310-7.
131. Martinez G, Pestana de Castro AF, Ribeiro Pagnani KJ, Nakazato G, Dias da Silveira W, Gottschalk M. Clonal distribution of an atypical MRP+, EF*, and suilysin+ phenotype of virulent *Streptococcus suis* serotype 2 strains in Brazil. Can J Vet Res. 2003;67(1):52-5.
132. Costa AT, Lobato FC, Abreu VL, Assis RA, Reis R, Uzal FA. Serotyping and evaluation of the virulence in mice of *Streptococcus suis* strains isolated from diseased pigs. Rev Inst Med Trop Sao Paulo. 2005;47(2):113-5.
133. Wei Z, Li R, Zhang A, He H, Hua Y, Xia J, et al. Characterization of *Streptococcus suis* isolates from the diseased pigs in China between 2003 and 2007. Vet Microbiol. 2009;137(1-2):196-201.
134. Li LL, Liao XP, Sun J, Yang YR, Liu BT, Yang SS, et al. Antimicrobial resistance, serotypes, and virulence factors of *Streptococcus suis* isolates from diseased pigs. Foodborne Pathog Dis. 2012;9(7):583-8.
135. Trottier S, Higgins R, Brochu G, Gottschalk M. A case of human endocarditis due to *Streptococcus suis* in North America. Rev Infect Dis. 1991;13(6):1251-2.

136. Fittipaldi N, Collis T, Prothero B, Gottschalk M. *Streptococcus suis* meningitis, Hawaii. Emerg Infect Dis. 2009;15(12):2067-9.
137. Nagel A, Manias V, Busquets N, Sniadowsky S, Anzardi J, Mendez Ede L. [*Streptococcus suis* meningitis in an immunocompetent patient]. Rev Argent Microbiol. 2008;40(3):158-60.
138. Nunez JM, Marcotullio M, Rojas A, Acuna L, Caceres M, Mochi S. [First case of meningitis by *Streptococcus suis* in the norwest area of Argentina]. Rev Chilena Infectol. 2013;30(5):554-6.
139. Demar M, Belzunce C, Simonnet C, Renaux A, Abboud P, Okandze A, et al. *Streptococcus suis* meningitis and bacteremia in man, French Guiana. Emerg Infect Dis. 2013;19(9):1545-6.
140. Vlieghe E. The microbiological spectrum of invasive bacterial infections in Cambodian adults and implications for standard treatment guidelines. Belgium: University of Leuven; 2014.
141. Cheng AF, Oo KT, Li EK, French GL. Septic arthritis caused by *Streptococcus suis* serotype 2. J Infect. 1987;14(3):237-41.
142. Hu X, Zhu F, Wang H, Chen S, Wang G, Sun J, et al. [Studies on human streptococcal infectious syndrome caused by infected pigs]. Zhonghua Yu Fang Yi Xue Za Zhi. 2000;34(3):150-2.
143. Feng Y, Shi X, Zhang H, Zhang S, Ma Y, Zheng B, et al. Recurrence of Human *Streptococcus suis* Infections in 2007: Three Cases of Meningitis and Implications that Heterogeneous *S. suis* 2 Circulates in China. Zoonoses Public Health. 2009;56(9-10):506-14.
144. Ho AK, Woo KS, Tse KK, French GL. Infective endocarditis caused by *Streptococcus suis* serotype 2. J Infect. 1990;21(2):209-11.
145. Woo J. *Streptococcus suis* meningitis in man in Hong Kong. Trans R Soc Trop Med Hyg. 1986;80(5):848-9.
146. Woo J, Li EK. *Streptococcus suis* meningitis requires prolonged treatment with penicillin. Infection. 1987;15(2):129-30.
147. Ibaraki M, Fujita N, Tada M, Ohtaki O, Nagai H. [A Japanese case of *Streptococcus suis* meningitis associated with lumbar epidural abscess]. Rinsho Shinkeigaku. 2003;43(4):176-9.
148. Matsuo H, Sakamoto S. [Purulent meningitis caused by *Streptococcus suis* in a pig breeder]. Kansenshogaku Zasshi. 2003;77(5):340-2.
149. Ishigaki K, Nakamura A, Iwabuchi S, Kodera S, Ooe K, Kataoka Y, et al. [A case of *Streptococcus suis* endocarditis, probably bovine-transmitted, complicated by pulmonary embolism and spondylitis]. Kansenshogaku Zasshi. 2009;83(5):544-8.
150. Mori K, Ishii N, Mochizuki H, Taniguchi A, Shiomi K, Nakazato M. Bilateral sensorineural hearing impairment due to *Streptococcus suis* meningitis 20 days after swine bite. Rinsho Shinkeigaku. 2013;53(9):732-5.
151. Tambyah PA, Kumarasinghe G, Chan HL, Lee KO. *Streptococcus suis* Infection Complicated by Purpura Fulminans and Rhabdomyolysis: Case Report and Review. Clin Infect Dis. 1997;24(4):710-2.
152. Chan YC, Wilder-Smith A, Ong BK, Kumarasinghe G, Wilder-Smith E. Adult community acquired bacterial meningitis in a Singaporean teaching hospital. A seven-year overview (1993-2000). Singapore Med J. 2002;43(12):632-6.

153. Tan JH, Yeh BI, Seet CS. Deafness due to haemorrhagic labyrinthitis and a review of relapses in *Streptococcus suis* meningitis. Singapore Med J. 2010;51(2):e30-3.
154. Kim H, Lee SH, Moon HW, Kim JY, Lee SH, Hur M, et al. *Streptococcus suis* causes septic arthritis and bacteremia: phenotypic characterization and molecular confirmation. Korean J Lab Med. 2011;31(2):115-7.
155. Huh HJ, Park KJ, Jang JH, Lee M, Lee JH, Ahn YH, et al. *Streptococcus suis* meningitis with bilateral sensorineural hearing loss. Korean J Lab Med. 2011;31(3):205-11.
156. Choi SM, Cho BH, Choi KH, Nam TS, Kim JT, Park MS, et al. Meningitis Caused by *Streptococcus suis*: Case Report and Review of the Literature. J Clin Neurol. 2012;8(1):79-82.
157. Oh YJ, Song SH. A Case of *Streptococcus suis* Infection Causing Pneumonia with Empyema in Korea. Tuberc Respir Dis. 2012;73(3):178-81.
158. Tsai HY, Liao CH, Liu CY, Huang YT, Teng LJ, Hsueh PR. *Streptococcus suis* infection in Taiwan, 2000-2011. Diagn Microbiol Infect Dis. 2012;74(1):75-7.
159. Hanterdsith B, Tharavichitkul P, Mahanupab P, Raksamat W. Postmortem diagnosis of sudden unexpected death from *Streptococcus suis* type 2 infection: a case report. J Forensic Leg Med. 2013;20(4):347-9.
160. Leelarasamee A, Nilakul C, Tien-Grim S, Srifuengfung S, Susaengrat W. *Streptococcus suis* toxic-shock syndrome and meningitis. J Med Assoc Thai. 1997;80(1):63-8.
161. Chotmongkol V, Janma J, Kawamatawong T. *Streptococcus suis* meningitis: report of a case. J Med Assoc Thai. 1999;82(9):922-4.
162. Teekakirikul P, Wiwanitkit V. *Streptococcus suis* infection: overview of case reports in Thailand. Southeast Asian J Trop Med Public Health. 2003;34 Suppl 2:178-83.
163. Wangkaew S, Chaiwarith R, Tharavichitkul P, Supparatpinyo K. *Streptococcus suis* infection: a series of 41 cases from Chiang Mai University Hospital. J Infect. 2006;52(6):455-60.
164. Wangsomboonsiri W, Luksananun T, Saksornchai S, Ketwong K, Sungkanuparph S. *Streptococcus suis* infection and risk factors for mortality. J Infect. 2008;57(5):392-6.
165. Rusmeechan S, Sribusara P. *Streptococcus suis* meningitis: the newest serious infectious disease. J Med Assoc Thai. 2008;91(5):654-8.
166. Navacharoen N, Chantharochavong V, Hanprasertpong C, Kangsanarak J, Lekagul S. Hearing and vestibular loss in *Streptococcus suis* infection from swine and traditional raw pork exposure in northern Thailand. J Laryngol Otol. 2009;123(8):857-62.
167. Fongcom A, Pruksakorn S, Netsirisawan P, Pongprasert R, Onsibud P. *Streptococcus suis* infection: a prospective study in northern Thailand. Southeast Asian J Trop Med Public Health. 2009;40(3):511-7.
168. Laohapensang K, Rutherford RB, Arworn S. Mycotic abdominal aortic aneurysm due to *Streptococcus suis*: a case report. Surg Infect. 2010;11(2):179-81.
169. Pachirat O, Taksinachanekit S, Mootsikapun P, Kerdsin A. Human *Streptococcus suis* Endocarditis: Echocardiographic Features and Clinical Outcome. Clin Med Insights Cardiol. 2012;6:119-23.
170. Hampson DJ, Trott DJ, Clarke IL, Mwaniki CG, Robertson ID. Population structure of Australian isolates of *Streptococcus suis*. J Clin Microbiol. 1993;31(11):2895-900.
171. Nga TV, Nghia HD, Tu le TP, Diep TS, Mai NT, Chau TT, et al. Real-time PCR for detection of *Streptococcus suis* serotype 2 in cerebrospinal fluid of human patients with meningitis. Diagn Microbiol Infect Dis. 2011;70(4):461-7.

172. Wertheim HF, Nguyen HN, Taylor W, Lien TT, Ngo HT, Nguyen TQ, et al. *Streptococcus suis*, an important cause of adult bacterial meningitis in northern Vietnam. PLoS One. 2009;4(6):e5973.
173. Taylor WR, Nguyen K, Nguyen D, Nguyen H, Horby P, Nguyen HL, et al. The spectrum of central nervous system infections in an adult referral hospital in Hanoi, Vietnam. PLoS One. 2012;7(8):e42099.
174. Ho Dang Trung N, Le Thi Phuong T, Wolbers M, Nguyen Van Minh H, Nguyen Thanh V, Van MP, et al. Aetiologies of central nervous system infection in Viet Nam: a prospective provincial hospital-based descriptive surveillance study. PLoS One. 2012;7(5):e37825.
175. Spiss HK, Kofler M, Hausdorfer H, Pfausler B, Schmutzhard E. [*Streptococcus suis* meningitis and neurophysiology of the acoustic system. First case report from Austria]. Nervenarzt. 1999;70(8):738-41.
176. Colaert J, Allewaert M, Magerman H, Vandeven J, Vandepitte J. *Streptococcus suis* meningitis in man. First reported observation in Belgium. Acta Clin Belg. 1985;40(5):314-7.
177. Cammaert T, Verstraete W, Baeck E. [Deafness-blindness caused by *Streptococcus suis* meningitis--epidemiology and rehabilitation]. Acta Otorhinolaryngol Belg. 1990;44(1):37-41.
178. Hantson P, Vekemans MC, Gautier P, Mahieu P, Sindic CJ, Guerit JM, et al. Fatal *Streptococcus suis* meningitis in man. Acta Neurol Belg. 1991;91(3):165-8.
179. Coolen L, Dens J, Baeck E, Claes C, Lins RL, Verbraeken H, et al. *Streptococcus suis* meningitis, permanent perceptive deafness and endophthalmitis. Intensive Care Med. 1989;15(8):545.
180. Perch B, Kjems E. Group R *Streptococcus* infections in man. JAMA. 1971;216(9):1484.
181. Koldkjaer O, Nielsen G. Haemolytic *Streptococcus* group R infection. Br Med J. 1972;3(5829):765.
182. Kjems E, Perch B. [Serious infections in man caused by group R streptococci]. Ugeskr Laeger. 1975;137(12):682-3.
183. Hedegaard SS, Zaccarin M, Lindberg J. [Septic arthritis caused by *Streptococcus suis*]. Ugeskr Laeger. 2013;175(22):1574-5.
184. Avril MF, Ghanassia JP, Leger JM, Bure A, Modai J. [Meningitis by group R *Streptococcus*. About two observations]. Med Maladies Infect. 1977;7(12):531-4.
185. Paul G, Ancelle JP, Lionsquy G, Brochard C, Brassac R, Nevot P. [Human meningitis by group R *Streptococcus*: an unknown anthropozoonosis?]. Med Maladies Infect. 1977;7(12):525-9.
186. Faucqueur B, Proust J. [*Streptococcus suis* meningitis. An occupational disease]. Presse Med. 1983;12(29):1821.
187. Desjars P, Reynaud AE, Tasseau F, Courtieu AL. [*Streptococcus suis* infections: about a meningitis in a slaughterhouse worker]. Med Maladies Infect. 1987;17(8-9):469-71.
188. Dupas D, Vignon M, Geraut C. *Streptococcus suis* meningitis. A severe noncompensated occupational disease. J Occup Med. 1992;34(11):1102-5.
189. Bezian MC, Diallo BS, Chachia A, Gabinski C, Beyloi J. [Purpura fulminans during *Streptococcus suis* meningitis and septicemia]. Med Maladies Infect. 1996;26(3):349-51.
190. Tayoro J, Besnier JM, Laudat P, Cattier B, Choutet P. Infective endocarditis due to *Streptococcus suis* serotype 2. Eur J Clin Microbiol Infect Dis. 1996;15(9):765-6.

191. Caumont H, Gerard N, Depernet B, Brasme L, Eschard JP, Etienne JC. [*Streptococcus suis* L3-L4 spondylodiscitis in a butcher]. Presse Med. 1996;25(29):1348.
192. Bouchaud L, Bourgain C, Aouar M, Grise G, Morcamp D, Deséchalliers JP. [*Streptococcus suis* type II meningitis. About a case]. Med Maladies Infect. 1997;27(3):317-8.
193. François B, Gissot V, Ploy MC, Vignon P. Recurrent septic shock due to *Streptococcus suis*. J Clin Microbiol. 1998;36(8):2395.
194. Durand F, Périno CL, Recule C, Brion JP, Kobish M, Guerber F, et al. Bacteriological diagnosis of *Streptococcus suis* meningitis. Eur J Clin Microbiol Infect Dis. 2001;20(7):519-21.
195. Bensaid T, Bonnefoi-Kyriacou B, Dupel-Pottier C, Bellon O, Lagier E, Chardon H. [*Streptococcus suis* meningitis following wild boar hunting]. Presse Med. 2003;32(23):1077-8.
196. Pedroli S, Kobisch M, Beauchet O, Chaussinand JP, Lucht F. [*Streptococcus suis* bacteremia]. Presse Med. 2003;32(13 Pt 1):599-601.
197. Fauveau L, Mourtada Y, Hazouard E. [Meningoencephalitis related-to *Streptococcus suis* in a butcher: relevance of occupational questionnaire at emergency room]. Ann Fr Anesth Reanim. 2007;26(9):814-5.
198. Bahloul H, Mofredj A, Mrabet A, Gineyt G, Rousselier P. [*Streptococcus suis* meningitis after oral contamination?]. Med Maladies Infect. 2008;38(5):281-2.
199. Kaufhold A, Lutticken R, Litterscheid S. [Systemic infection caused by *Streptococcus suis*]. Dtsch Med Wochenschr. 1988;113(42):1642-3.
200. Bungener W, Bialek R. Fatal *Streptococcus suis* septicemia in an abattoir worker. Eur J Clin Microbiol Infect Dis. 1989;8(4):306-8.
201. Grebe T, Bergenthal D, Fahr AM, Scheja HW. [Meningitis caused by *Streptococcus suis* type 2 in an adult]. Dtsch Med Wochenschr. 1997;122(41):1244-7.
202. Rosenkranz M, Elsner HA, Sturenburg HJ, Weiller C, Rother J, Sobottka I. *Streptococcus suis* meningitis and septicemia contracted from a wild boar in Germany. J Neurol. 2003;250(7):869-70.
203. Heidt MC, Mohamed W, Hain T, Vogt PR, Chakraborty T, Domann E. Human infective endocarditis caused by *Streptococcus suis* serotype 2. J Clin Microbiol. 2005;43(9):4898-901.
204. Braun S, Jechart G, Emmerling U, Ehret W. [Meningitis caused by *Streptococcus suis*]. Dtsch Med Wochenschr. 2007;132(20):1098-100.
205. Mazokopakis EE, Kofteridis DP, Papadakis JA, Gikas AH, Samonis GJ. First case report of *Streptococcus suis* septicaemia and meningitis from Greece. Eur J Neurol. 2005;12(6):487-9.
206. Voutsadakis IA. *Streptococcus suis* endocarditis and colon carcinoma: a case report. Clin Colorectal Cancer. 2006;6(3):226-8.
207. Baddeley PG. *Streptococcus suis* infection. Occup Med. 1995;45(4):222.
208. Manzin A, Palmieri C, Serra C, Saddi B, Princivalli MS, Loi G, et al. *Streptococcus suis* meningitis without history of animal contact, Italy. Emerg Infect Dis. 2008;14(12):1946-8.
209. Camporese A, Tizianel G, Bruschetta G, Cruciatti B, Pomes A. Human meningitis caused by *Streptococcus suis*: the first case report from north-eastern Italy. Infez Med. 2007;15(2):111-4.

210. Perseghin P, Bezzi G, Troupioti P, Gallina M. *Streptococcus suis* meningitis in an Italian blood donor. Lancet. 1995;346(8985):1305-6.
211. Kloppenburg M, Mulder NH, Houwerzijl J. Septicaemia due to porcine streptococci. Lancet. 1975;306(7946):1218.
212. Zanen HC, Engel HWB. Porcine streptococci causing meningitis and septicaemia in man. Lancet. 1975;305(7919):1286-8.
213. van Jaarsveld BC, van Kregten E, van Kesteren RG, Rozenberg-Arska M, Bartelink AK. [Fulminant sepsis caused by *Streptococcus suis*]. Ned Tijdschr Geneeskd. 1990;134(30):1462-4.
214. Bartelink AK, van Kregten E. *Streptococcus suis* as threat to pig-farmers and abattoir workers. Lancet. 1995;346(8991-8992):1707.
215. Halaby T, Hoitsma E, Hupperts R, Spanjaard L, Luirink M, Jacobs J. *Streptococcus suis* Meningitis, a Poacher's Risk. Eur J Clin Microbiol Infect Dis. 2000;19(12):943-5.
216. Peetermans WE, Moffie BG, Thompson J. Bacterial endocarditis caused by *Streptococcus suis* type 2. J Infect Dis. 1989;159(3):595-6.
217. Arend SM, van Buchem MA, van Ogtrop ML, Thompson J. Septicaemia, meningitis and spondylodiscitis caused by *Streptococcus suis* type 2. Infection. 1995;23(2):128.
218. van de Beek D, Spanjaard L, de Gans J. *Streptococcus suis* meningitis in the Netherlands. J Infect. 2008;57(2):158-61.
219. de Ceuster LM, van Dillen JJ, Wever PC, Rozemeijer W, Louwerse ES. [*Streptococcus suis* meningitis in a meat factory employee]. Ned Tijdschr Geneeskd. 2012;156(44):A5080.
220. Zalas-Wiecek P, Michalska A, Grabczewska E, Olczak A, Pawlowska M, Gospodarek E. Human meningitis caused by *Streptococcus suis*. J Med Microbiol. 2013;62(Pt 3):483-5.
221. Taipa R, Lopes V, Magalhaes M. *Streptococcus suis* meningitis: first case report from Portugal. J Infect. 2008;56(6):482-3.
222. Dragojlovic J, Milosevic B, Sasic N, Pelemis M, Sasic M. [*Streptococcus suis* infection--clinical manifestations]. Med Pregl. 2005;58(5-6):236-9.
223. Tarradas C, Luque I, de Andres D, Abdel-Aziz Shahein YE, Pons P, Gonzalez F, et al. Epidemiological relationship of human and swine *Streptococcus suis* isolates. J Vet Med B Infect Dis Vet Public Health. 2001;48(5):347-55.
224. Martinez Aviles P, Jusdado Ruiz-Capillas JJ, Gomez Rodrigo J, Solis Villa J. [Sacroiliitis caused by *Streptococcus suis* type II]. An Med Interna. 1994;11(6):309.
225. Juncal AR, Pardo F, Rodriguez I, Perez del Molino ML. [Meningitis by *Streptococcus suis*]. Enferm Infecc Microbiol Clin. 1997;15(2):120-1.
226. de la Hoz Adame ME, de la Rubia Martin F, Dominguez Fuentes B, Garcia Gil D. [Acute meningitis caused by *Streptococcus suis*: review of a case in a splenectomized patient]. An Med Interna. 2005;22(10):507.
227. Alonso-Socas Mdel M, Aleman-Valls R, Roldan-Delgado H, Gomez-Sirvent JL. [Endocarditis and spondylodiscitis caused by *Streptococcus suis*]. Enferm Infecc Microbiol Clin. 2006;24(5):354-5.
228. Luengo-Alvarez J, Martin-Ruiz C, Sanchez Munoz-Torrero JF, Iniguez-Ovando R. [Meningitis due to *Streptococcus suis*: a case report]. Enferm Infecc Microbiol Clin. 2006;24(5):352-4.

229. Hidalgo A, Ropero F, Palacios R, Garcia V, Santos J. Meningitis due to *Streptococcus suis* with no contact with pigs or porcine products. J Infect. 2007;55(5):478.
230. Riquelme E, Escribano E, Blanch JJ, Crespo MD. [Acute *Streptococcus suis* meningitis in a woman working in a meat market]. Enferm Infecc Microbiol Clin. 2008;26(4):256-7.
231. Aspiroz C, Vela AI, Pascual MS, Aldea MJ. [Acute infective endocarditis due to *Streptococcus suis* serotype 2 in Spain]. Enferm Infecc Microbiol Clin. 2009;27(6):370-1.
232. Galbarro J, Franco-Alvarez de Luna F, Cano R, Angel Castano M. [Acute meningitis and spondylodiscitis due to *Streptococcus suis* in a patient who had no contact with pigs or porcine products]. Enferm Infecc Microbiol Clin. 2009;27(7):425-7.
233. Fernandez-Ferro J, Lopez-Gonzalez FJ, Pardo F, Pias-Peleteiro JM. [Acute *Streptococcus suis* meningitis in a pig breeder]. Enferm Infecc Microbiol Clin. 2011;29(5):396-7.
234. Christensen P, Kronvall G. [A case of *Streptococcus suis* meningitis--a new occupational disease in Sweden?]. Lakartidningen. 1985;82(3):119.
235. Hickling P, Cormack FC. Meningitis caused by group R haemolytic streptococci. Br Med J. 1976;2(6047):1299-300.
236. Agass MJ, Willoughby CP, Bron AJ, Mitchell CJ, Mayon-White RT. Meningitis and endophthalmitis caused by *Streptococcus suis* type II (group R). Br Med J. 1977;2(6080):167-8.
237. McLendon BF, Bron AJ, Mitchell CJ. *Streptococcus suis* type II (group R) as a cause of endophthalmitis. Br J Ophthalmol. 1978;62(10):729-31.
238. Joynson DH. Infections of man and Group R *Streptococci*. Br J Clin Pract. 1980;34(5):147-9, 54.
239. Shneerson JM, Chattopadhyay B, Murphy MF, Fawcett IW. Permanent perceptive deafness due to *Streptococcus suis* type II infection. J Laryngol Otol. 1980;94(4):425-7.
240. Twort CH. Group R streptococcal meningitis (*Streptococcus suis* type II): a new industrial disease? Br Med J (Clin Res Ed). 1981;282(6263):523-4.
241. Clements MR, Hamilton DV, Clifton-Hadley FA, O'Reilly JF. *Streptococcus suis* type II infection. A new industrial disease? Practitioner. 1982;226(1364):323-5.
242. McNeil NI, Gordon T. Meningitis caused by *Streptococcus suis* type II. Postgrad Med J. 1986;62(730):743-4.
243. Hay PE, Cunniffe JG, Kramer G, France AJ, Gray JA, Watt B. Two cases of *Streptococcus suis* meningitis. Br J Ind Med. 1989;46(5):352-3.
244. Meecham JS, Worth RC. Persistent diplopia following *Streptococcus suis* type 2 meningitis. J R Soc Med. 1992;85(9):579-80.
245. Doube A, Calin A. Bacterial endocarditis presenting as acute monoarthritis. Ann Rheum Dis. 1988;47(7):598-9.
246. Maher D. *Streptococcus suis* septicaemia presenting as severe acute gastro-enteritis. J Infect. 1991;22(3):303-4.
247. Bronstein AM, Morland AB, Ruddock KH, Gresty MA. Recovery from bilateral vestibular failure: implications for visual and cervico-ocular function. Acta Otolaryngol Suppl. 1995;520 Pt 2:405-7.
248. Rao SS, Mariathas A, Teare L. Meningitis in a butcher. Emerg Med J. 2008;25(9):607-8.

249. Kennedy KJ, Jadeer AA, Ong CW, Senanayake SN, Collignon PJ. Two cases of *Streptococcus suis* endocarditis in Australian piggery workers. Med J Aust. 2008;189(7):413.
250. Tramontana AR, Graham M, Sinickas V, Bak N. An Australian case of *Streptococcus suis* toxic shock syndrome associated with occupational exposure to animal carcasses. Med J Aust. 2008;188(9):538-9.
251. Rehm T, Baums CG, Strommenger B, Beyerbach M, Valentin-Weigand P, Goethe R. Amplified fragment length polymorphism of *Streptococcus suis* strains correlates with their profile of virulence-associated genes and clinical background. J Med Microbiol. 2007;56(Pt 1):102-9.
252. Ye C, Zhu X, Jing H, Du H, Segura M, Zheng H, et al. *Streptococcus suis* sequence type 7 outbreak, Sichuan, China. Emerg Infect Dis. 2006;12(8):1203-8.

Tables

Table 1. Worldwide distribution of serotypes for reported clinical *S. suis* cases of infection in pigs by country from January 1st, 2002 to December 31st, 2013.

Country	Clinical Cases	Predominant Serotypes[1] (Frequency in %)			References
WORLDWIDE	4711	2 (27.9%)	9 (19.4%)	3 (15.9%)	
NORTH AMERICA	3 162 (67.1%)	2 (24.3%)	3 (21.0%)	1/2 (13.0%)	
Canada	3 065	2	3	1/2	(25, 29)
United States	97	3	2	7	(130)
SOUTH AMERICA	125 (2.7%)	2 (57.6%)	1/2 (9.6%)	14 (8.8%)	
Brazil	125	2	1/2	14	(131, 132)
ASIA	659 (14.0%)	2 (44.2%)	3 (12.4%)	4 (5.6%)	
Mainland China	639	2	3	4	(30, 112, 133, 134)
South Korea	20	3	4	2, 8, 22	(68)
EUROPE	765 (16.2%)	9 (61.0%)	2 (18.4%)	7 (6.7%)	
Netherlands	99	9	2	7	(77)
Spain	666	9	2	7	(56, 75, 76)

[1] Only the three most predominant serotypes, which were identified by coagglutination (or an equivalent method using reference antisera), are shown in this table.

Table 2. Worldwide distribution of reported clinical *S. suis* cases of infection in humans by country until December 31st, 2013.

Country	Reported Cases	Confirmed Serotypes[1]			Unconfirmed[3] or Unknown Serotypes[4]	References
		2	14	Others[2]		
WORLDWIDE	1 642	1 227 (74.7%)	33 (2.0%)	5 (0.3%)	377 (23.0%)	
NORTH AMERICA	8 (0.5%)	7 (87.5%)	1 (12.5%)	0	0	
Canada	5	4	1	-	-	(7, 20, 100, 135)
United States	3	3	-	-	-	(121, 122, 136)
SOUTH AMERICA	9 (0.5%)	2 (22.2%)	0	1 (11.1%)	6 (66.7%)	
Argentina	4	1	-	1	2	(64, 67, 137, 138)
Chile	4	-	-	-	4	(65, 66)
French Guiana	1	1	-	-	-	(139)
ASIA	1 481 (90.2%)	1 133 (76.5%)	29 (2.0%)	3 (0.2%)	316 (21.3%)	
Cambodia	13	13	-	-	-	(140)
Mainland China	245	245	-	-	-	(16, 90, 91,

						141-143)
Hong Kong	69	53	-	-	16	(12, 73, 92, 93, 107, 144-146)
Japan	11	10	-	-	1	(118, 147-150)
Laos	1	-	-	-	1	(Unpubl.[5])
Philippines (USA)	1	-	-	-	1	(94)
Singapore	3	-	-	-	3	(151-153)
South Korea	4	-	-	-	4	(154-157)
Taiwan	7	2	2	-	3	(21, 22, 158)
Thailand	553	292	21	2	238	(11, 13, 23, 26, 45, 63, 88, 110, 115, 119, 120, 159-169)
Vietnam	574	518	6	1	49	(10, 62, 89, 170-174)
EUROPE	140 (8.5%)	84 (60.0%)	3 (2.1%)	1 (0.7%)	52 (37.1%)	
Austria	1	-	-	-	1	(175)
Belgium	4	3	-	-	1	(176-179)
Croatia	2	-	-	-	2	(44)
Denmark	8	6	1	-	1	(9, 98, 180-183)
France	19	8	-	-	11	(95-97, 184-198)
Germany	9	8	-	-	1	(19, 86, 108, 199-204)
Greece	2	-	-	-	2	(205, 206)

Ireland	1	-	-	-	1	(207)
Italy	3	2	-	-	1	(208-210)
Netherlands	51	39	1	1	10	(14, 211-219)
Poland	1	-	-	-	1	(220)
Portugal	1	-	-	-	1	(221)
Serbia	5	-	-	-	5	(222)
Spain	13	4	-	-	9	(125, 223-233)
Sweden	1	1	-	-	-	(234)
United Kingdom	19	13	1	-	5	(99, 235-248)
OCEANIA	4 (0.2%)	1 (25.0%)	0	0	3 (75.0%)	
Australia	3	-	-	-	3	(249, 250)
New Zealand	1	1	-	-	-	(85)

[1] Serotypes were identified by coagglutination (or an equivalent method using reference antisera) or by PCR specific reaction for serotype 2 (and 1/2) or for serotype 14 (and 1).

[2] Serotypes other than serotypes 2 and 14. See main text and Table 3 for details.

[3] Serotypes were determined based on biochemical identification so are reported as "unconfirmed"

[4] Strain serotype was not mentioned in the publication and is thus considered as "unknown".

[5] (H. Wertheim, 2014, personal communication)

Table 3. Clinical manifestations of reported clinical *S. suis* cases of infection in humans by serotypes confirmed by coagglutination.

Confirmed Serotypes	Reported Cases	Clinical Manifestations			
		Meningitis	Septic Shock[1]	Other[2]	Unknown[3]
2	867	590 (68.1%)	233 (26.9%)	25 (2.9%)	19 (2.2%)
14	28	14 (50.0%)	6 (21.4%)	-	8 (28.6%)
4	1	1	-	-	-
5	1	-	-	1	-
16	1	-	-	1	-
21	1	1	-	-	-
24	1	-	1	-	-

[1] Septic shock includes bacteremia, sepsis, septicemia, and streptococcal toxic shock-like syndrome cases.

[2] Other clinical manifestations such as endocarditis, septic arthritis, pneumonia, peritonitis, pulmonary edema, myocarditis, etc.

[3] Clinical manifestations were either not specified or could not be associated with a particular serotype.

Table 4. Studies exploring human carriage and exposure to *S. suis* in risk groups.

Study (Reference)	Technique Employed/ Target	Risk Groups	Positive Carriage or Exposure [Positive/Tested]	Serotypes Found
Serological				
New Zealand, 1989 [(103)]	Indirect ELISA / Serotype 2 whole bacteria	Pig farmers	15/70 (21.4%)	-
		Meat inspectors	11/107 (10.3%)	
		Dairy farmers	9/96 (9.4%)	
Netherlands, 1999 [(105)]	Western Blot / Serotype 2 MRP/EF	Veterinarians	MRP: 6/100 (6.0%) EF: 2/100 (2.0%)	-
		Pig farmers	MRP: 2/190 (1.1%) EF: 1/190 (0.5%)	
United States, 2008 [(104)]	Indirect ELISA / Serotype 2 whole bacteria	Pig workers	7/73 (9.6%)	-
Upper Respiratory Tract Colonization (Isolation)				
Italy, 1989 [(106)]	Tonsil swabs	Slaughterhouse workers	2/10 (20%)	2
Mexico, 2001 [(101)]	Tonsil swabs	Slaughterhouse workers	4/69 (5.8%)	2, 27, NT[1]
Germany, 2002 [(108)]	Pharyngeal swabs	Slaughtering and meat processing	7/132 (5.3%)	2

workers

[1] Non-typable strain (NT).

Table 5. Determined sequence types of reported clinical *S. suis* cases of infection in pigs from January 1st, 2002 to December 31st, 2013.

Country	Serotypes[1]	ST	ST Complex	Number of Cases	References
NORTH AMERICA					
Canada	2	1	1	2	(124)
		25	25	28	(52, 124)
		28	28	18	(52)
		145	25	1	MLST Database
	23	80	Unrelated	1	(51)
	24	68	Unrelated	1	(51)
	25	69	Unrelated	1	(51)
	27	72	Unrelated	1	(51)
	28	75	Unrelated	1	(51)
	29	92	Unrelated	1	(51)
	30	77	Unrelated	1	(51)
	31	70	Unrelated	1	(51)
	Not Specified	13	Unrelated	Not Specified	(51)
United States	2	1	1	3	(52)
		25	25	28	(52)
		28	28	33	(52)
EUROPE					
Denmark	4	54	53/54	1	(51)
	5	53	53/54	1	(51)
	6	55	Unrelated	1	(51)

	8	87	87	1	(51)
	9	82	Unrelated	1	(51, 124)
	10	78	Unrelated	1	(51)
	11	91	Unrelated	1	(51)
	12	74	Unrelated	1	(51)
	13	71	Unrelated	1	(51, 123)
	16	73	Unrelated	1	(51)
Finland	2	37	Unrelated	1	(51)
	Not Specified	27	27	Not Specified	(51)
		38	Unrelated	1	(51)
France	2	1	1	4	(116)
		140	Unrelated	1	MLST Database
		141	1	1	MLST Database
		142	28	1	MLST Database
		143	1	1	MLST Database
		144	1	1	MLST Database
		229	25	1	MLST Database
		230	25	1	MLST Database
	14	231	Unrelated	1	MLST Database
	15	81	Unrelated	1	MLST Database
Germany	1/2	100	Unrelated	1	(251)
	2	1	1	5	(251)
		25	25	1	(251)
		28	28	2	(251)
		97	Unrelated	1	(251)
	3	95	Unrelated	1	(251)
	7	29	29	7	(124, 251)
		89	16	1	(251)
	9	93	Unrelated	1	(251)
		96	Unrelated	1	(251)

		98	16	2	(251)
		99	16	1	(251)
Italy	2	1	1	1	(80)
	9	138	Unrelated	1	MLST Database
Netherlands	1	1	1	4	(77, 124)
		13	13/149	2	(51, 77)
		149	13/149	1	(77)
		156	1	1	(77)
	1/2	1	1	1	(77, 124)
	2	1	1	21	(77, 124)
		20	147	19	(77)
		29	29	1	(77)
		134	1	1	MLST Database
		146	1	1	MLST Database
	3	15	16	1	(77)
		35	27	1	(51)
	4	17	147	3	(77)
	7	29	29	5	(77)
		135	29	1	MLST Database
		136	16	1	(77)
		150	Unrelated	1	(77)
		153	Unrelated	1	MLST Database
		218	Unrelated	1	(77)
	8	87	87	1	(77)
		198	16	1	(77)
	9	1	1	2	(77, 124)
		15	16	1	(77)
		16	16	38	(77)
		136	16	2	(77)
		148	1	1	(77)

		151	16	1	(77)
		152	16	1	MLST Database
		154	16	1	MLST Database
		155	16	1	(77)
		182	Unrelated	1	(77)
		184	Unrelated	1	MLST Database
		189	Unrelated	1	(77)
		220	Unrelated	1	(77)
	15	81	Unrelated	1	(51)
	Not Specified	183	Unrelated	1	MLST Database
Spain	1	1	1	2	(111)
					MLST Database
	1/2	1	1	2	(111)
					MLST Database
		28	28	1	MLST Database
		64	94	1	(51)
	2	1	1	31	(111)
		5	1	1	(51)
		27	27	1	MLST Database
		28	28	1	MLST Database
		86	1	1	MLST Database
		124	1	1	(111)
	3	14	147	1	(51)
		15	16	1	(51)
		27	27	1	MLST Database
		89	16	1	(51)
	4	16	16	1	(51, 124)
	5	17	147	1	(51)
	9	16	16	2	(51, 124)
		59	123	1	(51)

		123	123	8	(111)
		125	123	9	(111)
		361	Unrelated	1	MLST Database
		367	Unrelated	1	MLST Database
	14	1	1	1	(111) MLST Database
	15	65	Unrelated	2	(51)
	27	65	Unrelated	2	(51)
United Kingdom	1	1	1	5	(116, 124) MLST Database
		12	11	1	(51)
		13	13/149	1	(51)
	1 or 14	10	1	1	(51)
	2	1	1	37	(116, 124) MLST Database
		2	1	5	(51)
		9	1	1	(51)
		25	25	7	MLST Database
		28	28	5	MLST Database
		29	29	1	MLST Database
		30	28	1	MLST Database
	3	27	27	2	(51)
		29	29	1	MLST Database
		31	28	2	(51)
		33	27	1	(51)
		42	Unrelated	1	(51)
	4	23	87	3	(51)
	5	39	Unrelated	1	MLST Website
		44	Unrelated	1	(51)
	7	29	29	6	MLST Database

		34	225	1	(51)
		83	29	1	(51)
	8	1	1	1	MLST Database
	9	46	Unrelated	1	(51)
		48	Unrelated	2	(51)
		50	Unrelated	1	(51)
	11	88	Unrelated	1	(51)
	14	1	1	14	MLST Database
	15	43	43/52	1	(51)
		45	Unrelated	1	(51)
		49	Unrelated	1	(51)
		52	43/52	1	(51)
	16	41	Unrelated	2	(51) MLST Website
		47	Unrelated	1	(51)
	28	21	87	1	(51)
	Not Specified	40	Unrelated	1	MLST Website
		51	Unrelated	1	MLST Website
		90	Unrelated	1	MLST Website
ASIA					
Mainland China	2	1	1	49	(112, 113, 127)
		7	7	216	(112, 113, 116, 123, 127, 252)
		25	25	1	(113)
		28	28	31	(112, 113, 127)
		86	1	1	(127)
		117	27	27	MLST Database
		162	28	1	(127)
		223	1	1	MLST Database

	228	7	1	MLST Database
	242	1	1	MLST Database
	244	7	1	MLST Database
	245	28	1	MLST Database
	289	1	1	(112)
	290	Unrelated	1	MLST Database
	352	Unrelated	1	MLST Database
	353	Unrelated	1	MLST Database
	354	Unrelated	1	MLST Database
	355	Unrelated	1	MLST Database
	418	Unrelated	1	MLST Database
	419	Unrelated	1	MLST Database
3	224	Unrelated	1	MLST Database
7	129	29	1	MLST Database
	225	225	1	MLST Database
	335	Unrelated	1	MLST Database
	420	Unrelated	1	MLST Database
9	222	Unrelated	1	MLST Database
	226	226/227	1	MLST Database
	227	226/227	1	MLST Database
	239	239/241	1	MLST Database
	241	239/241	1	MLST Database
	417	Unrelated	1	MLST Database
11	260	Unrelated	1	MLST Database
	263	Unrelated	1	MLST Database
13	262	Unrelated	1	MLST Database
27	258	Unrelated	1	MLST Database
31	261	27	1	MLST Database
	265	27	1	MLST Database
Not Specified	29	29	1	(112)

		118	Unrelated	1	(112)
		156	1	2	(112)
		264	Unrelated	1	MLST Database
		266	Unrelated	1	MLST Database
		267	Unrelated	1	MLST Database
		303	Unrelated	1	MLST Database
		383	Unrelated	2	MLST Database
		421	Unrelated	1	MLST Database
		422	Unrelated	1	MLST Database
Japan	1	1	1	1	(53, 114)
	2	1	1	5	(53, 114)
		28	28	48	(53, 114)
		324	28	1	(114)
	3	108	94	1	(53)
		117	27	1	(53)
	7	29	29	1	(53)
		118	Unrelated	1	(53)
	11	108	94	1	(53)
Vietnam	9	390	Unrelated	1	MLST Database

[1] Serotypes were identified by coagglutination (or an equivalent method using reference antisera), by PCR or, sometimes, by undefined methods.

Table 6. Determined sequence types of reported clinical *S. suis* cases of infection in humans from January 1st, 2002 to December 31st, 2013.

Country	Serotype[1]	ST	ST Complex	Number of Cases	References
NORTH AMERICA					
Canada	2	25	25	3	(53), (116)
	14	6	1	1	(51)
United States	2	1	1	1	(53)
		25	25	1	(121)
SOUTH AMERICA					
Argentina	2	1	1	1	(Unpubl.[2])
French Guiana	2	1	1	1	(139)
EUROPE					
France	2	20	147	2	(53), (116)
Italy	2	1	1	1	(80)
		134	1	2	(80), (208)
Netherlands	2	1	1	14	(77), (124)
		20	147	11	(77), (124)
		134	1	1	(77)
		146	1	1	(77)
	14	6	1	1	(51)
Spain	2	3	1	1	(125)
United Kingdom	2	1	1	1	(53)
	14	2	1	1	MLST Database
ASIA					
Cambodia	2	1	1	13	(140)
Mainland China	2	1	1	11	(116), (252)

		7	7	210	(116), (124), (252)
	14	1	1	1	(123)
Hong Kong	2	1	1	14	(51), (117)
		9	1	12	(51), (117)
		25	25	1	(117)
Japan	2	1	1	7	(53), (118)
		28	28	1	(118)
Thailand	2	1	1	123	(53)
		25	25	17	(53), (115)
		28	28	4	(53), (115)
		101	225	1	(119)
		102	25	2	(53)
		103	25	6	(53), (115)
		104	225	45	(53)
		126	1	3	(115)
	5	181	Unrelated	1	(88)
	14	11	11	1	(53)
		105	1	19	(110, 120)
		127	1	1	(120)
	24	221	221/234	1	(88)
Vietnam	2	1	1	56	(10, 124)
		107	1	1	(10)
	14	105	1	1	(10)
	16	106	Unrelated	1	(89)

[1] Serotypes were identified by coagglutination (or an equivalent method using reference antisera) or by PCR. However, it is impossible to distinguish between serotypes 1 and 14 and serotypes 1/2 and 2 by PCR, so serotypes remain to be confirmed.
[2] (M. Gottschalk, 2014, unpubl. data)

Figures

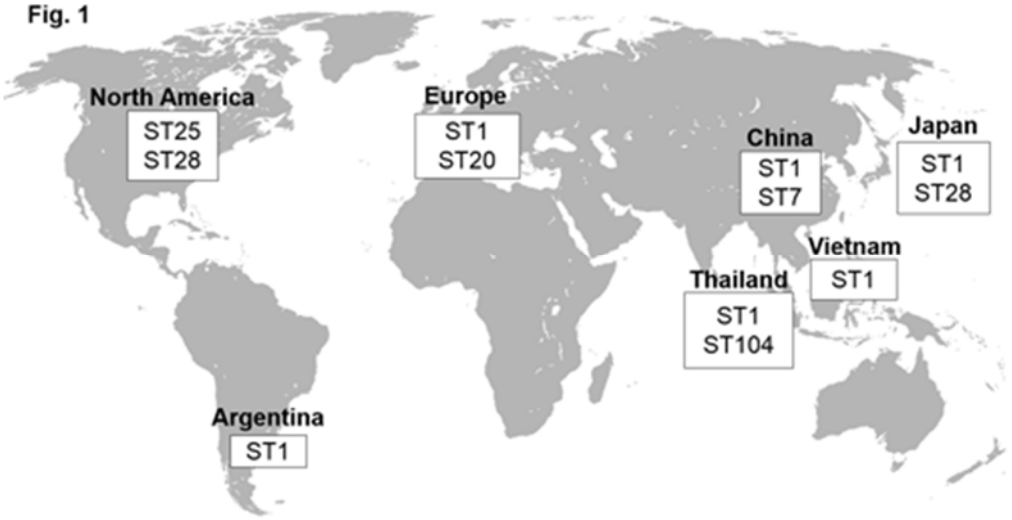


Figure 1. Worldwide distribution of the most important *S. suis* serotype 2 sequence types (STs) isolated from both clinical pig and human cases of infection.

ARTICLE II

Streptococcus suis serotype 3 and serotype 18 capsular polysaccharides contain di-N-acetyl-bacillosamine

---

Guillaume Goyette-Desjardins [1,2], Evgeny Vinogradov [2,3], Masatoshi Okura [4], Daisuke Takamatsu [4,5], Marcelo Gottschalk [1,2], and Mariela Segura [1,2]

[1] Swine and Poultry Infectious Diseases Research Center, Faculty of Veterinary Medicine, University of Montreal, 3200 Sicotte St., St-Hyacinthe, Quebec, Canada, J2S 2M2

[2] Canadian Glycomics Network (GlycoNet), University of Alberta, 11227 Saskatchewan Dr., Edmonton, Alberta, Canada, T6G 2G2

[3] National Research Council, 100 Sussex Dr., Ottawa, Ontario, Canada, K1A 0R6

[4] Division of Bacterial and Parasitic Disease, National Institute of Animal Health, National Agriculture and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

[5] The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1193, Japan

Publié dans *Carbohydrate Research.* **2018**. 466:18-29.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai participé activement à la conception de l'étude, aux expériences (production et purification des polysaccharides; 30%), à l'analyse des résultats et à l'écriture de l'article.

Abstract

Streptococcus suis serotype 3 is counted among the *S. suis* serotypes causing clinical disease in pigs. Yet, limited information is available on this serotype. Here we determined for the first time the chemical composition and structure of serotype 3 capsular polysaccharide (CPS), a major bacterial virulence factor and the antigen at the origin of *S. suis* classification into serotypes. Chemical and spectroscopic data gave the repeating unit sequence for serotype 3: [4)D-GlcA(β1-3)D-QuiNAc4NAc(β1-]$_n$. To the best of our knowledge, this is the first report of di-N-acetyl-D-bacillosamine (QuiNAc4NAc) containing polysaccharides in *Streptococci* and the second time this rare diamino sugar has been observed in a Gram-positive bacterial species since its initial report. This led to the identification of homologues of UDP-QuiNAc4NAc synthesis genes in *S. suis* serotype 18. Thus, the repeating unit sequence for serotype 18 is: [3)D-GalNAc(α1-3)[D-Glc(β1-2)]D-GalA4OAc(β1-3)D-GalNAc(α1-3)D-QuiNAc4NAc(α1-]$_n$. A correlation between *S. suis* serotypes 3 and 18 CPS sequences and genes of these serotypes' *cps* loci encoding putative glycosyltransferases and polymerase responsible for the biosynthesis of the repeating unit was tentatively established. Knowledge of CPS structure and composition will contribute to better dissect the role of this bacterial component in the pathogenesis of *S. suis* serotypes 3 and 18.

1. Introduction

Streptococcus suis is an encapsulated Gram-positive bacterium and an important swine pathogen. It is responsible for important economic losses to the swine industry worldwide. Striking manifestations of the disease in pigs are septicemia and meningitis, but can also be present as endocarditis, pneumonia, and arthritis. The natural habitat of *S. suis* is the upper respiratory tract of pigs, more particularly the tonsils and nasal cavities, but also the genital and digestive tracts. Almost 100% of pig farms worldwide have carrier animals, although only a limited number of pigs develop clinical disease in the presence of preventive medication (1). Yet, recent implementation of compulsory reduction in the use of antibiotics in livestock production has led to a worriedly increase of clinical cases due to *S. suis* in pigs. *S. suis* is also an emerging zoonotic agent, being responsible for septicemia with or without septic shock, meningitis and other less common infections usually related to generalized septicemia. This zoonosis is of particular importance worldwide to people in close contact with infected pigs and/or pork-derived products. Though human infections in Western countries are considered an occupational disease (such as veterinarians and abattoir workers), in some Asian countries, the general population is at risk since many cases of infection have been reported after ingestion of contaminated raw pork products.

Cell-associated capsular polysaccharide (CPS) is considered to be one of *S. suis* most important virulence factor (2, 3). In fact, the originally described 35 serotypes of *S. suis* are based upon CPS antigenicity, serotype 2 being generally considered the most virulent and prevalent worldwide. The latest review analyzing the distribution of *S. suis* serotypes in diseased pigs with data from studies published between 2002 to 2013 reported serotype 3 to be the third predominant serotype worldwide with 16.4% of reported clinical cases (4). Yet, distribution varies geographically and in time. That same review reported that in North America, serotype 3 is almost equally as prevalent as serotype 2 (21.0% and 24.3%, respectively), while in Asia it is found second to serotype 2 with about 12.4% of cases (4). However, since the publication of that review, other epidemiological reports were published. Studies on *S. suis* serotype distribution from diseased pigs in the province of Québec, Canada, reported a steady decrease in serotype 3 prevalence from 14.5% to 9.4% between 2008 and

2011 (5) that was sustained over 2012-2014 with a prevalence of 7.0% for the 3-year period (6). Nonetheless, serotype 3 remains so far the third most prevalent serotype isolated in that region after serotypes 2 and 1/2 (6). As for Asia, a study on diseased pigs isolates, from 14 provinces in central and eastern regions of China between 2008 and 2010, reported serotype 3 as the second most prevalent after serotype 2 (7). Another epidemiological study on *S. suis* serotype distribution among isolates from pig carcasses sold in wet markets in Chiang Mai Province, northern Thailand, reported that serotype 3 was the most prevalent (8). Additionally, a recent study with isolates collected throughout Korea between 2009-2010 reported serotype 3 as the most prevalent in both slaughtered and diseased pigs with 15.8%, followed closely by serotype 2 with 15.0% (9). Although serotype 3 has so far not been isolated from human patients, an eventual role in zoonosis cannot be completely ruled out, especially when considered the popularity of local raw pork dishes in South-East Asia (4, 10).

In spite of the aforementioned epidemiological importance of *S. suis* serotype 3, the molecular aspects of the pathogenesis of the disease induced by this serotype and the involved virulence factors are largely unknown compared to the extensively studied serotype 2 (11). In fact, knowledge on the contribution of CPS structural diversity to *S. suis* pathogenesis, from colonization to infection of its host, is required in order to help devise new control strategies against *S. suis* infections. Due to its importance, ongoing research yielded complete structural determination for the CPSs of *S. suis* serotype 2 (12), serotype 14 (13), serotypes 1 & 1/2 (14) and serotype 9 (15). In this work, we aimed to describe for the first time the primary structure of the CPS polymer for the reference strain of *S. suis* serotype 3 and to assign the functions of the *cps* locus genes involved in its biosynthesis.

2. Material and Methods

2.1. Capsular polysaccharide production, purification and quality controls

S. suis serotype 3 reference strain 4961 (16) and serotype 18 field strain 1734119 (isolated from a diseased pig in Quebec, Canada – our collection) were used in this study. For the CPS production, 6 L of fresh Todd-Hewitt Broth (Oxoid, Thermo Fisher Scientific, Nepean, Canada) were inoculated and grown overnight as previously described (15).

Three methods of CPS extraction were compared. **Method A** was based on previous publications (12, 15). Briefly, bacterial cells from the 6-L culture were pelleted by centrifugation at 10,000*g* for 40 min, suspended by repeated pipetting in a buffer containing 33 mmol/L phosphate and 145 mmol/L NaCl pH 8.0, and chilled. The bacterial suspension was autoclaved at 121°C for 15 min. The supernatant containing the crude CPS was recovered by centrifugation at 9,000*g* for 50 min. For **Method B**, the bacterial pellet from the 6-L culture was resuspended in deionized water (ddH_2O). The bacteria were killed by heating at 60 °C for 45 min, which has been confirmed by the absence of growth on blood agar plates. Finally, the content of the tubes was lyophilized for 72 h. Then, bacterial cells were stirred with ddH_2O (3 g dry cells in 100 mL) overnight at room temperature (15). The supernatant containing the crude CPS was recovered by centrifugation at 9,000*g* for 50 min. For **Method C**, the bacterial pellet from the 6-L culture was resuspended in ddH_2O, heat-killed, and lyophilized as described above (Method B). Bacterial cells were then boiled with 2% AcOH (3 g dry cells in 100 mL) for 5 min. The supernatant containing the crude CPS was neutralized with NaOH, then recovered by centrifugation at 9,000*g* for 50 min.

The crude CPSs were further purified from the extracted material by solvent extraction, precipitations, and gel filtration as previously described in (12, 15) with some modifications. For gel filtration chromatography, a XK-26/100 column filled with Sephacryl S-400 HR (GE Healthcare, Uppsala, Sweden) was used and eluted with 50 mmol/L NH_4HCO_3 (for serotype 3) or with 50 mmol/L NaCl (for serotype 18) at a flow rate of 1.3 ml/min, using an ÄKTA Purifier 10 system (GE Healthcare), including a UV-900 Monitor, and equipped with a Knauer Smartline 2300 RI Detector (Knauer, Berlin, Germany) connected to the system via an AD-900 Analog/Digital Converter (GE Healthcare). Fractions were collected and assayed for CPS by dot-blot with a serotype specific anti-*S. suis* rabbit polyclonal antibody as the primary antibody and horseradish-peroxidase-conjugated (HRP-conjugated) goat anti-rabbit secondary antibody (17). Fractions giving a positive RI signal and response with antibodies but no absorption at 280 and 254 nm were pooled and freeze dried. The purified material was dissolved in water, dialyzed against ddH_2O for 24 h at 4°C, and finally freeze dried.

Quality controls for presence of contaminating nucleic acids and proteins were performed as previously described (12-14). The calculated limit of detection ($P \leq 0.05$) for the modified Lowry protein assay was 9 μg/ml. Size-exclusion chromatography coupled with multi-angle light scattering (SEC–MALS) experiments were also performed, essentially as previously described (13).

2.2. NMR spectroscopy

NMR experiments were carried out on a Bruker AVANCE III 600 MHz (^{1}H) spectrometer with 5 mm Z-gradient probe with acetone internal reference (2.225 ppm for ^{1}H and 31.45 ppm for ^{13}C) using standard pulse sequences cosygpprqf (gCOSY), mlevphpr (TOCSY) (mixing time 120 ms), roesyphpr (ROESY) (mixing time 500 ms), hsqcedetgp (HSQC), and hmbcgplpndqf (HMBC) (100 ms long range transfer delay). Resolution was kept < 3 Hz/pt in F2 in H-H correlations and < 5 Hz/pt in F2 of H-C correlations. Number of points in F2 set so to have AQ about 0.8 s for H-H correlations, 0.24 s for C-H HSQC. Number of points in F1 was 1/4 of F2. The spectra were processed and analyzed using the Bruker Topspin 2.1 program.

2.3. General methods

Gel chromatography was performed on Sephadex G-15 column (1.5x60 cm) or Sephadex G-50 column (2.5x60 cm) in 1% acetic acid and elution was monitored using a refractive index detector (Gilson). For 2D NMR, desalting was performed on Sephadex G15.

2.4. Depolymerization by methanolysis (OS, serotype 3)

Polysaccharide sample (5 mg) was depolymerized with 1 mL of 1 M HCl/MeOH (90°C, 1 h). Sample was dried by an air stream and purified by gel-chromatography on Sephadex G-15 column.

2.5. Alkaline treatment (DPS, serotype 18)

Polysaccharide sample (5 mg) was dissolved in 1 mL of 12% NH_4OH and heated (70 °C, 20 min). Sample was dried by an air stream.

2.6. Oxidation treatment (DPS-ox, serotype 18)

Deacetylated polysaccharide sample (5 mg) was dissolved in water (2 mL), $NaIO_4$ (20 mg) was added, and the solution was kept for 24 h at room temperature. Then, ethylene glycol (0.2 mL) and an excess of $NaBD_4$ were added to the solution for 1 h, followed by treatment with AcOH (0.2 mL) and desalted on Sephadex G-15 column. Product was hydrolyzed with 1 ml of 2% AcOH (100°C, 2 h), separated on Sephadex G-50 column to give oxidized polysaccharide.

2.7. Determination of neutral and amino sugars as alditol acetates

Monosaccharides were detected as reduced and acetylated derivatives (alditol acetates). Polysaccharide sample (0.2 mg) with inositol internal standard were hydrolyzed with 1 mL of 3 M trifluoroacetic acid (120 °C, 3 h). The sample was then dried, reduced with $NaBD_4$, and reagent destroyed with 0.5 mL of AcOH. The obtained solution was dried under an air stream, dried twice with addition of MeOH (1 mL), acetylated with 0.4 mL Ac_2O-0.4 mL pyridine for 30 min at 100 °C, dried, and finally analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) (Thermo ion-trap instrument, capillary column DB-17, 160-260°C by 4°/min).

2.8. Absolute configurations

For the determination of absolute configuration, to the polysaccharides (0.3 mg) were added either (R)- or racemic-2-butanol (0.2 mL) and acetyl chloride (20 μL) at room temperature, heated at 100 °C for 2 h, dried by air stream, acetylated (0.2 mL Ac_2O-0.2 mL pyridine, 100 °C, 30 min), dried, and finally analyzed by GC-MS as described above.

3. Results

3.1. Capsular polysaccharide production and purification for serotype 3

Using the reference strain for *S. suis* serotype 3 (#4961), 29-30 mg of CPS were obtained after extraction (Method A) and purification by gel filtration from 6 L of fermentation broth in two different experiments. Nucleic acid analysis confirmed the absence

of significant contamination giving a range of 0.1%-0.7% w/w DNA/RNA. Similarly, proteins levels were found to be very low (0.5% w/w) in the purified CPS. For serotype 3, weight average molar weights (M_W) of 1.169 x 10^5 g/mol was determined for the native polysaccharide by SEC-MALS.

3.2. Structure determination and NMR for serotype 3

The purified serotype 3 CPS was analyzed by 2D NMR using COSY, TOCSY, ROESY, ^{1}H-^{13}C HSQC, and HMBC experiments. Monosaccharides were identified by COSY, TOCSY and NOESY cross peak patterns and ^{13}C NMR chemical shifts (Fig. 1 and Table 1). Aminogroups location was concluded from high field signal position of aminated carbons (CH at 45-60 ppm) and both were found acetylated. Spectra contained signals of disaccharide repeating units made from β-GlcA and α-2,4-diacetamido-2,4,6-trideoxy-D-glucose (Di-*N*-acetyl-D-bacillosamine or QuiNAc4NAc, unit A). All sugars were in pyranose form. Identity and absolute configuration of D-GlcA were determined by GC-MS of 2-butyl glycoside-ester acetates with optically pure 2-butanol. According to the calculation of ^{13}C chemical shifts (18), QuiNAc4NAc also had a D-configuration. All sugars had NMR signal positions and coupling constants in agreement with the presented structure:

$$[4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow3)\text{-}\beta\text{-D-Qui}p\text{NAc4NAc-}(1\rightarrow]_n$$

B A

The sequence of the monosaccharides was determined from the following NOE and HMBC inter-residual correlations: A1:B4 and B1:A3. In addition, CPS was depolymerized by methanolysis and produced a disaccharide

β-GlcA6OMe-3-α,β-QuiNAc4NAc-OMe

B A

with α:β ratio of 1:4. It was analyzed by NMR in the same way as the PS (Table 1). These results confirmed the proposed structure for the serotype 3 CPS.

3.3. Identification of other di-N-acetyl-bacillosamine-containing serotypes

Di-*N*-acetyl-D-bacillosamine (QuiNAc4NAc; also known as 2,4-diacetamido-2,4,6-trideoxy-D-glucose or $BacNAc_2$) is a rare diamino-sugar first described in 1957 in a polysaccharide isolated from Gram-positive *Bacillus subtilis* strain later renamed *Bacillus licheniformis* (19, 20). So far, QuiNAc4NAc and its derivatives have been reported at the terminal non-reducing end of *N*-linked (*Campylobacter jejuni*) and *O*-linked (*Neisseria gonorrhoeae*) glycoproteins (19, 21), but also in the LPS core or *O*-antigens from many (19) strains of Gram-negative bacteria (see Supplementary Table 1). This makes QuiNAc4NAc one of the few prokaryote-specific sugar, and an interesting target for the development of alternatives to antibiotics. The biosynthetic pathway for the conversion of UDP-GlcNAc into UDP-QuiNAc4NAc was biochemically studied in *C. jejuni* (PglF, PglE and PglD), *N. gonorrhoeae* (PglD, PglC and acetyltransferase domain of PglB) and *Acinetobacter baumannii* (WeeK, WeeJ and WeeI) (reviewed in (21)). The first step in the pathway is performed by a membrane-bound NAD^+-dependent dehydratase catalyzing C4 oxidation of GlcNAc, which also promotes elimination of water across C5 and C6, yielding an UDP-4-keto-6-deoxy sugar. The second step is catalyzed by a pyridoxal ’5’-phosphate (PLP)-dependent aminotransferase that catalyzes the transfer of the amino group from L-glutamate to the C4 position of the UDP-4-keto-6-deoxy-GlcNAc. The third and final step in bacillosamine biosynthesis is performed by an acetyl coenzyme A (AcCoA)-dependent acetyltransferase that catalyzes the C4 *N*-acetylation of the UDP-4-amino-6-deoxy-GlcNAc to yield UDP-QuiNAc4NAc.

The surprising discovery of QuiNAc4NAc in *S. suis* serotype 3 CPS led us to firstly investigate whether homologues of UDP-QuiNAc4NAc synthesis gene products of *C. jejuni*, *N. gonorrhoeae*, and/or *A. baumannii* are present in serotype 3 *cps* locus (22). Among *cps* gene products encoded by *S. suis cps3* gene cluster (22), Cps3E, Cps3G, and Cps3I showed 37.2%, 38.6%, and 39.1% amino acid identities to PglF, PglE, and PglD of *C. jejuni*, respectively (Table 2). These Cps3 products also shared 27.8% to 40.7% identities with UDP□QuiNAc4NAc synthesis gene products/domain of *N. gonorrhoeae* and *A. baumannii* (Table 2). Therefore, Cps3E, Cps3G, and Cps3I were considered to be dehydrogenase, aminotransferase and acetyltransferase, respectively, which are required for the synthesis of UDP-QuiNAc4NAc in *S. suis* serotype 3 reference strain.

We then compared UDP-QuiNAc4NAc synthesis gene products of *C. jejuni*, *N. gonorrhoeae*, *A. baumannii* (Table 2) and *S. suis* serotype 3 (Table 3) with *cps* gene products of other *S. suis* serotype reference strains. Interestingly, only serotypes 18 and 13 reference strains had homologues of all the three UDP-QuiNAc4NAc synthesis gene products (Tables 2 and 3). However, the putative acetyltransferase of serotype 13 (Cps13N) showed lower similarity with those of *C. jejuni* and *N. gonorrhoeae* than Cps3I and Cps18I (Table 2). Furthermore, Cps13N was also similar to the *O*-acetyltransferase for sialic acid synthesis (NeuD; 33-35% similarity with Cps1S, Cps2R, Cps1/2R, Cps6S, Cps14R, and Cps16T), and *cps13* gene cluster additionally includes other putative sialic acid synthesis genes (*cps13O, cps13P and cps13Q* encoding NeuB, NeuA and NeuC homologues, respectively) (22), implying that Cps13N is not involved in UDP-QuiNAc4NAc synthesis. Based on these results, we predicted that *S. suis* serotype 18 is likely to have QuiNAc4NAc in its CPS, while it might be present or not in the CPS of serotype 13. Therefore, it was also decided to describe for the first time the primary structure of the CPS polymer for the *S. suis* serotype 18 and to assign the functions of the *cps* locus genes involved in its biosynthesis.

3.4. Capsular polysaccharide production and purification for serotype 18

Using a well encapsulated strain of *S. suis* serotype 18 (1734119), 6-7 mg of high-molecular weight CPS was obtained after extraction with Method A and purification by gel filtration from 6 L of fermentation broth in two different experiments. Low CPS yields can be explained by the high degree of depolymerization as suggested by the observation of multiple peaks eluting from the gel filtration column (data not shown). Degradation is most likely related to the autoclave step, as it has been previously observed with *S. suis* serotype 9 CPS (15) and led to complicated spectra (data not shown).

In order to improve purification and analysis, we tried extracting CPS with water (Method B), as it was the case with *S. suis* serotype 9 CPS (15), though this method resulted in less than 1 mg of the serotype 18 CPS after purification from 6 L of fermentation broth. We then opted to boil bacterial cells in 2% acetic acid for 5 min (Method C), yielding 6 mg from a single CPS peak from the gel filtration column, suggesting that this polysaccharide might be covalently bound to the cells. This CPS fraction gave a strong reaction with the serotype 18

specific rabbit anti-*S. suis* serum by dot-blot. Nucleic acid analysis confirmed the absence of significant contamination with 0.5% w/w DNA/RNA. Similarly, proteins levels were found to be low (1.6% w/w) in the purified CPS.

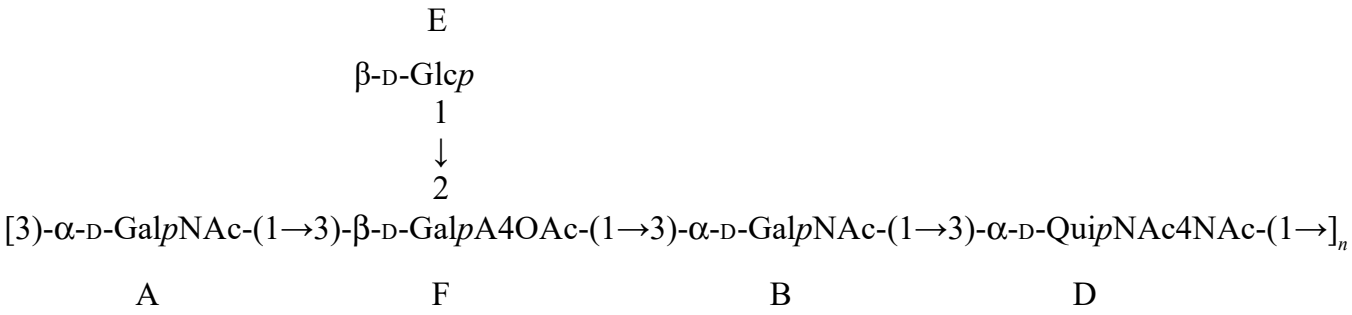
3.5 Structure determination and NMR for serotype 18

Polysaccharide (purified by Method C) was analyzed by 2D NMR using COSY, TOCSY, ROESY, and ^{1}H-^{13}C HSQC experiments. Monosaccharides were identified by COSY, TOCSY and NOESY cross peak patterns and ^{13}C NMR chemical shifts (Fig. 2 and Table 4). Aminogroup location was concluded from high field signal position of aminated carbons (CH at 45-60 ppm) and were found acetylated by the signals position and corresponding number of N-acetyl signals. Spectra contained signals of pentasaccharide repeating units made from α-GalNAc, β-GalA4OAc, β-Glc, and α-QuiNAc4NAc. All sugars were in pyranose form, as followed from signal positions and coupling constants, characteristic for gluco- and galacto- pyranoses. GalNAc, GalA, and Glc were identified by GC-MS. D-GalNAc, D-GalA, and D-Glc absolute configurations were determined by GC-MS of 2-butyl glycosides-esters acetates with optically pure 2-butanol. All sugars had NMR signal positions and coupling constants in agreement with the presented structure. The sequence of the monosaccharides was determined from the following NOE and HMBC inter-residual correlations: A1:F3; B1:D3; D1:A3; E1:F2; F1:B3.

Deacetylation of the CPS by alkaline treatment resulted in deacylated PS, which was analyzed in the same way by NMR and the results are shown in Table 4. The main difference in the spectra of native and deacylated PS was in the position of H/C-4 signal of the β-GalA F, shifted from 5.78/68.4 ppm in the native PS to 4.53/66.1 ppm in the spectra of deacylated PS.

Oxidation of the serotype 18 O-deacylated PS gave a linear PS with tetrasaccharide repeating units due to removal of side glucose E. This caused changes in the position of the signals of originally branched β-GalA F, particularly H/C-2 shifted from 4.08/74.3 ppm to 3.61/70.0 ppm.

Taken together, these results yielded the following pentasaccharide repeating unit for the serotype 18 CPS:



3.6 Assignment of functions of the cps locus genes involved in serotypes 3 and 18 CPS biosynthesis

The serotype 3 and 18 *cps* gene clusters were previously characterized [GenBank accession nos. BR001001 (serotype 3) and AB737825 (serotype 18)] [22]. From the above section 3.3, three gene products of each serotype (Cps3E, Cps3G, Cps3I, Cps18E, Cps18G, and Cps18I) were considered to be linked to QuiNAc4NAc synthesis. We further tentatively assigned functions of other *cps* genes involved in serotypes 3 and 18 CPS biosynthesis on the basis of the CPS structures determined in this study and sequence homologies of putative *cps3* and *cps18* gene products (Tables 5 and 6 and Fig. 3).

In *C. jejuni*, in addition to PglF, PglE and PglD required for the biosynthesis of UDP-QuiNAc4NAc, other *pgl* gene products are involved in the synthesis of QuiNAc4NAc-containing *N*-linked glycan (23). The glycan donor for the *N*-linked glycosylation is an undecaprenyl pyrophosphate (Und-PP)-linked heptasaccharide: D-Gal*p*NAc(α1-4)D-Gal*p*NAc(α1-4)[D-Glc*p*(β1-3)]D-Gal*p*NAc(α1-4)D-Gal*p*NAc(α1-4)D-Gal*p*NAc(α1-3)D-Qui*p*NAc4NAc-(α1)-PP-Und (24). In the assembly pathway of the Und-PP linked heptasaccharide, the QuiNAc4NAc 1-phosphoryl transferase PglC transfers the first sugar QuiNAc4NAc to undecaprenyl phosphate to form Und-PP-QuiNAc4NAc. Then, PglA adds a GalNAc residue to Und-PP-QuiNAc4NAc, PglJ adds the second GalNAc residue, PglH adds the next three GalNAc residues, and finally PglI adds the single branching Glc residue (23, 25, 26).

Among the *cps* gene products of serotypes 3 and 18, the putative initial glycosyltransferases (Cps3H and Cps18H, respectively) were highly similar to each other (amino acid identity, 96.6%; coverage, 100%). In addition, both Cps3H and Cps18H showed more than 60% amino acid identities to PglC of *C. jejuni* ATCC 700819 (Tables 5 and 6). As PglC is the initial glycosyltransferase for *N*-linked glycan synthesis in *C. jejuni*, which transfers QuiNAc4NAc-phosphate to undecaprenyl phosphate carrier, the homology search results suggested that QuiNAc4NAc is the initial sugar of the repeating units transferred by Cps3H and Cps18H in both serotypes (Fig. 3). QuiNAc4NAc is the only sugar residue commonly present in both serotypes 3 and 18 CPSs. This fact and the almost identical amino acid sequences between Cps3H and Cps18H may further support the above hypothesis.

Except for Cps3H, the serotype 3 *cps* gene cluster encodes only one putative glycosyltransferase Cps3J (Table 5). Therefore, Cps3J was predicted to be associated with the addition of GlcA to QuiNAc4NAc, although we could not find homologous glycosyltransferases that are known to catalyze this reaction in the publicly available database (Table 5 and Fig. 3A). Based on the conserved domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), Cps3K and Cps3L were predicted to be the flippase Wzx involved in the export of repeating units and the polymerase Wzy involved in the polymerization of CPS, respectively (Table 5). Cps3N, which had a domain of UDP-glucose dehydrogenase family, showed similarity with UDP-glucose 6-dehydrogenase Ugd of *Streptococcus pneumoniae* (Table 5). Ugd is known to catalyze UDP-GlcA synthesis (27), and *S. pneumoniae* strains, which encode Ugd in the *cps* gene clusters, possess GlcA in the CPS (28). Therefore, Cps3N was assigned to UDP-glucose dehydrogenase related to UDP-GlcA synthesis.

The repeating unit of the serotype 18 CPS is composed of five sugars. This indicates that serotype 18 *cps* gene cluster encodes four glycosyltransferases required for the repeating unit assembly in addition to the initial glycosyltransferase. Cps18J, Cps18K, and Cps18L were predicted to be glycosyltransferases by the conserved domain search (Table 6), but none of the other *cps18* gene products possessed the glycosyltransferase domain, and thus another glycosyltransferase was not found by the search. However, Cps18M was similar to putative

glycosyltransferase WcyD of *S. pneumoniae* (amino acid identity, 40-41%; coverage, 96-99%) (Table 6). Although the function of WcyD is unknown, we regarded Cps18M as one of the glycosyltransferases required for the repeating unit assembly in serotype 18. Among the four putative glycosyltransferases, Cps18J showed some similarity with PglA of *C. jejuni* ATCC 700819 (amino acid identity, 26.4%; coverage, 91.2%). As PglA is the glycosyltransferase that adds a GalNAc residue to Und-PP-QuiNAc4NAc in *C. jejuni*, this result suggests that Cps18J is responsible for the addition of the second sugar GalNAc to QuiNAc4NAc (Table 6 and Fig. 3B). However, we could not predict the functions of other three glycosyltransferases convincingly by the blast search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Cps18K and Cps18L were partially similar to PglI (similarity, 34.8%; coverage, 29.4%) and PglA (similarity, 28.7%; coverage, 25.0%) of *C. jejuni* ATCC 700819, respectively (Table 6). As described above, PglI and PglA add the single branching Glc residue and a GalNAc residue, respectively, in the *N*-linked glycan synthesis of *C. jejuni*; therefore, Cps18K and Cps18L might catalyze the transfer of the single branching Glc residue and another GalNAc residue, respectively, in the serotype 18 CPS repeating unit. If that is the case, the remaining putative glycosyltransferase Cps18M might add the GalA4-OAc residue to GalNAc, although mutational and *in vitro* analyses are necessary to confirm these hypotheses. By the blast search, we assigned Cps18N and Cps18O to polymerase Wzy and flippase Wzx, respectively (Table 6). From the CPS18 structure, the presence of GalA4-OAc synthesis genes in the *cps18* gene cluster was implied. We predicted that Cps18P, Cps18Q, and Cps18R are involved in GalA4-OAc synthesis, because Cps18Q and Cps18R are similar to epimerase and dehydrogenase related to the synthesis of UDP-GalA of *S. pneumoniae* (Table 6) (28) and Cps18P had similarity with a putative acetyltransferase of *S. pneumoniae* (Table 6).

4. Discussion

This report is the first description of the sugar sequence of *S. suis* serotype 3 and serotype 18 CPSs. Serotype 3 repeating unit (RU) was found to be much simpler than those previously described for other *S. suis* serotypes, being an acidic linear disaccharide composed of D-GlcA and QuiNAc4NAc. On the other hand, serotypes 2, 14, 1, and 1/2 RUs are formed of acidic branched hexa- or heptasaccharides and composed of D-Glc, D-GlcNAc, D-Gal, D-

GalNAc, L-Rha, and with *N*-acetylneuraminic acid (Neu5Ac) at their non-reducing ends as a common feature (Supplementary Fig. 1). Serotype 9 RU is formed of an acidic branched tetrasaccharide composed of D-Gal, L-Rha, glucitol, 2-acetamido-2,6-dideoxy-β-D-*xylo*-hexopyranos-4-ulose (6d*xyl*Hex*p*NAc-4-ulo), and phosphate (Supplementary Fig. 1). Serotype 18 RU is formed of an acidic branched pentasaccharide composed of D-Glc, D-GalNAc, D-GalA4OAc, and D-QuiNAc4NAc. Although the structure of *S. suis* serotype 13 CPS was not elucidated in this study, it would be interesting to perform it in a future structural study of *S. suis* CPSs.

Release of CPS from the bacteria of previously reported *S. suis* serotypes has always been relatively easy to perform by either autoclaving (types 2, 14, 1, and 1/2) (12-14) or extracting with water (type 9) (15). These observations led to the hypothesis that these polysaccharides are not covalently bound to the cells (15). It was also the case in this study with serotype 3 CPS that was extracted by autoclaving. However, as herein reported for serotype 18, the polysaccharide extracted by autoclaving presented significant degradation by gel filtration chromatography and complicated NMR spectra with multiple amine-like signals that were removed by alkaline treatment. Furthermore, extracting with water yielded almost no material. Thus, this may be the first example of a *S. suis* cell-anchored CPS. Based on a previous putative assignment of the CPS synthesis genes that suggested that serotype 18 CPS gene cluster might contain a glycosyl-1-phosphate transferase gene (22) and the possibility that this base-labile 1-phosphate might be located in the backbone of the polysaccharide, we opted for a weak acid extraction of the CPS that yielded enough material to perform structural analyses. Now that we know that serotype 18 CPS does not contain phosphates, a base hydrolysis could possibly yield reasonable amounts. As a matter of fact, base hydrolysis to release CPS has been shown to be highly efficient with Group B *Streptococcus*, a bacterium well known for harboring covalently surface-anchored CPSs (29, 30).

Structurally, serotype 3 CPS is closely related to hyaluronic acid (also known as hyaluronan), which is widely distributed in mammal connective, epithelial and neural tissues: [4) D-GlcA(β1-3) D-Glc*p*NAc-(β1-]$_n$. The sole difference between these two polysaccharides is that *S. suis* serotype 3 CPS is composed of QuiNAc4NAc, which is biosynthetically derived

from GlcNAc. Hyaluronic acid was also described as a component of CPSs from Gram-positive streptococci (*S. pyogenes*, *S. equisimilis*, *S. uberis*, and *S. zooepidemicus*) and in Gram-negative type A *Pasteurella multocida* (31). Hyaluronic acid polysaccharides are produced in a synthase-dependent manner (31, 32). This synthase-dependent polymerization is sequentially catalyzed by the glycosyltransferases and produces relatively simple structures with only one or two repeating sugars. The synthase-dependent polysaccharide is made as a free glycan (not attached to a protein or lipid), without the need for a primer (*de novo* synthesis starts in the presence of the UDP-sugars and required cofactors) and is exported to the outside through a pore formed by the membrane-associated enzymatic complex. In contrast, CPS polymerization for all serotypes of *S. suis*, including serotype 3 "hyaluronic acid-like" CPS, is thought to proceed in a Wzy-dependent manner (22, 32). Polysaccharide synthesis is initiated by transfer of a nucleotide-activated sugar onto a lipid-carrier (Und-PP) at the inner side of the membrane by the initial glycosyltransferase, followed by the specific glycosyltransferases building sequentially the repeating unit block, which is then transported to the exterior by the flippase (Wzx) when fully formed. Once at the cell surface, the polymerase (Wzy) transfers the elongating polysaccharide chain from its lipid-carrier onto newly flipped repeating units. The CPS is attached to the cell wall by a complex of Wze/Wzd, and the lipid-carrier is released to be used again. Interestingly, a precursor of QuiNAc4NAc can be found as the initiating sugar (linked to the lipid-carrier) in *Streptococcus pneumoniae* serotype 5 CPS, namely 4-keto-*N*-acetyl-D-quinovosamine, which corresponds to the first intermediate in the QuiNAc4NAc biosynthesis pathway (28).

To our knowledge, this is the first report of di-*N*-acetyl-D-bacillosamine (QuiNAc4NAc) containing polysaccharides in *Streptococci* and the second time in a Gram-positive bacterial species since the initial report of this rare diamino sugar (20) (see Supplementary Table 1). It is also the third report of this sugar in a bacterial CPS, the first being of *Alteromonas sp.* strain CMM 155, a proteobacteria found in sea water (33) and the second being of *Colwellia psychrerythraea* strain 34H, a Gram-negative bacterium isolated from Arctic marine sediments (34). While it is still unknown why some bacteria decorate their surface-associated glycans with bacillosamine, it has been suggested that this sugar may serve

as a decoy to mammalian host immune systems and glycan-degrading enzymes (21, 35), since this sugar is unique.

Acknowledgements

The authors thank Ms. Claudia Duquette, Évelyne Lacasse and Élise Brouqui for excellent technical assistance during capsular polysaccharide production and purification. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a grant to MS (#342150) and partial support by the Fonds de recherche du Québec—Nature et technologies (FRQ-NT) (grant # 2018-PR-206110 to MS and MG). GGD is a recipient of a FRQNT Doctoral Scholarship and an Alexander Graham Bell Graduate Scholarship—Doctoral Program from NSERC.

References

1. Gottschalk M. Streptococcosis. In: Zimmerman JJ, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of swine. 10th ed. Ames, USA: Wiley-Blackwell Publishing; 2012. p. 841-55.
2. Gottschalk M, Segura M. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet Microbiol. 2000;76(3):259-72.
3. Roy D, Auger JP, Segura M, Fittipaldi N, Takamatsu D, Okura M, et al. Role of the capsular polysaccharide as a virulence factor for *Streptococcus suis* serotype 14. Can J Vet Res. 2015;79(2):141-6.
4. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect. 2014;3(6):e45.
5. Gottschalk M, Lacouture S, Bonifait L, Roy D, Fittipaldi N, Grenier D. Characterization of *Streptococcus suis* isolates recovered between 2008 and 2011 from diseased pigs in Quebec, Canada. Vet Microbiol. 2013;162(2-4):819-25.
6. Gottschalk M, Lacouture S. Canada: Distribution of *Streptococcus suis* (from 2012 to 2014) and *Actinobacillus pleuropneumoniae* (from 2011 to 2014) serotypes isolated from diseased pigs. Can Vet J. 2015;56(10):1093-4.
7. Chen L, Song Y, Wei Z, He H, Zhang A, Jin M. Antimicrobial susceptibility, tetracycline and erythromycin resistance genes, and multilocus sequence typing of *Streptococcus suis* isolates from diseased pigs in China. J Vet Med Sci. 2013;75(5):583-7.

8. Wongsawan K, Gottschalk M, Tharavichitkul P. Serotype- and virulence-associated gene profile of *Streptococcus suis* isolates from pig carcasses in Chiang Mai Province, Northern Thailand. J Vet Med Sci. 2015;77(2):233-6.
9. Oh SI, Jeon AB, Jung BY, Byun JW, Gottschalk M, Kim A, et al. Capsular serotypes, virulence-associated genes and antimicrobial susceptibility of *Streptococcus suis* isolates from pigs in Korea. J Vet Med Sci. 2017;79(4):780-7.
10. Gottschalk M, Xu J, Lecours M-P, Grenier D, Fittipaldi N, Segura M. *Streptococcus suis* Infections in Humans: What is the prognosis for Western countries? (Part II). Clin Microbiol Newsl. 2010;32(13):97-102.
11. Fittipaldi N, Segura M, Grenier D, Gottschalk M. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent *Streptococcus suis*. Future Microbiol. 2012;7(2):259-79.
12. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. Biochem Cell Biol. 2010;88(3):513-25.
13. Van Calsteren MR, Gagnon F, Calzas C, Goyette-Desjardins G, Okura M, Takamatsu D, et al. Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide. Biochem Cell Biol. 2013;91(2):49-58.
14. Van Calsteren MR, Goyette-Desjardins G, Gagnon F, Okura M, Takamatsu D, Roy R, et al. Explaining the serological characteristics of *Streptococcus suis* serotypes 1 and 1/2 from their capsular polysaccharide structure and biosynthesis. J Biol Chem. 2016;291(16):8387-98.
15. Vinogradov E, Goyette-Desjardins G, Okura M, Takamatsu D, Gottschalk M, Segura M. Structure determination of *Streptococcus suis* serotype 9 capsular polysaccharide and assignment of functions of the cps locus genes involved in its biosynthesis. Carbohydr Res. 2016;433:25-30.
16. Perch B, Pedersen KB, Henrichsen J. Serology of capsulated streptococci pathogenic for pigs: six new serotypes of *Streptococcus suis*. J Clin Microbiol. 1983;17(6):993-6.
17. Higgins R, Gottschalk M. An update on *Streptococcus suis* identification. J Vet Diagn Invest. 1990;2(3):249-52.
18. Lipkind GM, Shashkov AS, Knirel YA, Vinogradov EV, Kochetkov NK. A computer-assisted structural analysis of regular polysaccharides on the basis of 13C-n.m.r. data. Carbohydr Res. 1988;175(1):59-75.
19. Sharon N. Celebrating the golden anniversary of the discovery of bacillosamine, the diamino sugar of a *Bacillus*. Glycobiology. 2007;17(11):1150-5.
20. Sharon N. Polysaccharide containing amino-sugar from *Bacillus subtilis*. Nature. 1957;179(4566):919.
21. Morrison MJ, Imperiali B. The Renaissance of Bacillosamine and Its Derivatives: Pathway Characterization and Implications in Pathogenicity. Biochemistry. 2014;53(4):624-38.
22. Okura M, Takamatsu D, Maruyama F, Nozawa T, Nakagawa I, Osaki M, et al. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: potential mechanisms for generation of capsular variation. Appl Environ Microbiol. 2013;79(8):2796-806.
23. Glover KJ, Weerapana E, Chen MM, Imperiali B. Direct biochemical evidence for the utilization of UDP-bacillosamine by PglC, an essential glycosyl-1-phosphate transferase

in the *Campylobacter jejuni* N-linked glycosylation pathway. Biochemistry. 2006;45(16):5343-50.
24. Young NM, Brisson J-R, Kelly J, Watson DC, Tessier L, Lanthier PH, et al. Structure of the N-Linked Glycan Present on Multiple Glycoproteins in the Gram-negative Bacterium, *Campylobacter jejuni*. J Biol Chem. 2002;277(45):42530-9.
25. Glover KJ, Weerapana E, Imperiali B. *In vitro* assembly of the undecaprenylpyrophosphate-linked heptasaccharide for prokaryotic N-linked glycosylation. Proc Natl Acad Sci U S A. 2005;102(40):14255-9.
26. Linton D, Dorrell N, Hitchen PG, Amber S, Karlyshev AV, Morris HR, et al. Functional analysis of the *Campylobacter jejuni* N-linked protein glycosylation pathway. Mol Microbiol. 2005;55(6):1695-703.
27. Druzhinina TN, Kusov YY, Shibaev VN, Kochetkov NK, Biely P, Kucar S, et al. Uridine diphosphate 2-deoxyglucose. Chemical synthesis, enzymic oxidation and epimerization. Biochim Biophys Acta. 1975;381(2):301-7.
28. Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, Collins M, et al. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet. 2006;2(3):e31.
29. Michon F, Blake M, inventors; Baxter International, Inc., assignee. Procedures for the extraction and isolation of bacterial capsular polysaccharides for use a vaccines or linked to proteins as conjugate vaccines. United States patent US 6,248,570 B1. 2001 Jun. 19, 2001.
30. Calzas C, Goyette-Desjardins G, Lemire P, Gagnon F, Lachance C, Van Calsteren MR, et al. Group B *Streptococcus* and *Streptococcus suis* capsular polysaccharides induce chemokine production by dendritic cells via Toll-like receptor 2- and MyD88-dependent and -independent pathways. Infect Immun. 2013;81(9):3106-18.
31. Weigel PH, DeAngelis PL. Hyaluronan Synthases: A Decade-plus of Novel Glycosyltransferases. J Biol Chem. 2007;282(51):36777-81.
32. Yother J. Capsules of *Streptococcus pneumoniae* and Other Bacteria: Paradigms for Polysaccharide Biosynthesis and Regulation. Annu Rev Microbiol. 2011;65(1):563-81.
33. Zubkov VA, Nazarenko EL, Gorshkova RP, Ivanova EP, Shashkov AS, Knirel YA, et al. Structure of the capsular polysaccharide from *Alteromonas sp.* CMM 155. Carbohydr Res. 1995;275(1):147-54.
34. Casillo A, Stahle J, Parrilli E, Sannino F, Mitchell DE, Pieretti G, et al. Structural characterization of an all-aminosugar-containing capsular polysaccharide from *Colwellia psychrerythraea* 34H. Antonie Van Leeuwenhoek. 2017;110(11):1377-87.
35. Moran AP, Prendergast MM. Molecular Mimicry in *Campylobacter jejuni* and *Helicobacter pylori* Lipopolysaccharides: Contribution of Gastrointestinal Infections to Autoimmunity. J Autoimmun. 2001;16(3):241-56.
36. Haseley SR, Holst O, Brade H. Structural and serological characterisation of the O-antigenic polysaccharide of the lipopolysaccharide from *Acinetobacter haemolyticus* strain ATCC 17906. Eur J Biochem. 1997;244(3):761-6.
37. MacLean LL, Vinogradov E, Crump EM, Perry MB, Kay WW. The structure of the lipopolysaccharide O-antigen produced by *Flavobacterium psychrophilum* (259-93). Eur J Biochem. 2001;268(9):2710-6.
38. Vinogradov E, Perry MB. Characterisation of the core part of the lipopolysaccharide O-antigen of *Francisella novicida* (U112). Carbohydr Res. 2004;339(9):1643-8.

39. Hermansson K, Perry MB, Altman E, Brisson JR, Garcia MM. Structural studies of the O-antigenic polysaccharide of *Fusobacterium necrophorum*. Eur J Biochem. 1993;212(3):801-9.
40. Valueva OA, Zdorovenko EL, Varbanets LD, Shubchinskiy VV, Shashkov AS, Knirel YA. Structural studies of the O-polysaccharide of *Pragia fontium* 97U124 containing 2-acetamido-2,4,6-trideoxy-4-(D-glyceroyl)amino-D-glucose. Carbohydr Res. 2012;355:96-9.
41. Yokota S, Kaya S, Araki Y, Ito E. Structure of the O-polysaccharide chain of lipopolysaccharide from *Pseudomonas aeruginosa* IID 1001 (ATCC 27577). J Biochem. 1988;104(4):671-8.
42. Knirel YA, Vinogradov EV, Shashkov AS, Wilkinson SG, Tahara Y, Dmitriev BA, et al. Somatic antigens of *Pseudomonas aeruginosa*. The structure of O-specific polysaccharide chains of the lipopolysaccharides from *P. aeruginosa* O1 (Lanyi), O3 (Habs), O13 and O14 (Wokatsch), and the serologically related strain NCTC 8505. Eur J Biochem. 1986;155(3):659-69.
43. Hanniffy OM, Shashkov AS, Senchenkova SN, Tomshich SV, Komandrova NA, Romanenko LA, et al. Structure of a highly acidic O-specific polysaccharide of lipopolysaccharide of *Pseudoalteromonas haloplanktis* KMM 223 (44-1) containing L-iduronic acid and D-QuiNHb4NHb. Carbohydr Res. 1998;307(3-4):291-8.
44. Knirel' Iu A, Zdorovenko GM, Veremeichenko SI, Lipkind GM, Shashkova AS. [Antigenic polysaccharides of bacteria. 31. The structure of the O-specific polysaccharide chain of *Pseudomonas aurantiaca* IMB 31 lipopolysaccharide]. Bioorg Khim. 1988;14(3):352-8.
45. Zdorovenko GM, Veremeichenko SN, Zakharova I. [Immunochemical characteristics of a lipopolysaccharide from *Pseudomonas aurantiaca*]. Mikrobiologiia. 1988;57(6):1024-30.
46. Molinaro A, Evidente A, Sante Iacobellis N, Lanzetta R, Lo Cantore P, Mancino A, et al. O-Specific chain structure from the lipopolysaccharide fraction of *Pseudomonas reactans*: a pathogen of the cultivated mushrooms. Carbohydr Res. 2002;337(5):467-71.
47. Komandrova NA, Kokoulin MS, Kalinovsky AI, Tomshich SV, Romanenko LA, Vaskovsky VE. The O-specific polysaccharide of the marine bacterium *Rheinheimera pacifica* KMM 1406^T containing D- and L-2-acetamido-2-deoxy-galacturonic acids. Carbohydr Res. 2014;394:1-6.
48. Vinogradov E, MacLean LL, Brooks BW, Lutze-Wallace C, Perry MB. Structure of the O-polysaccharide of the lipopolysaccharide produced by *Taylorella asinigenitalis* type strain (ATCC 700933). Biochem Cell Biol. 2008;86(3):278-84.
49. Chowdhury TA, Jansson PE, Lindberg B, Lindberg J, Gustafsson B, Holme T. Structural studies of the *Vibrio cholerae* O:3 O-antigen polysaccharide. Carbohydr Res. 1991;215(2):303-14.
50. Hermansson K, Jansson P-E, Holme T, Gustavsson B. Structural studies of the *Vibrio cholerae* O:5 O-antigen polysaccharide. Carbohydr Res. 1993;248:199-211.
51. Kocharova NA, Perepelov AV, Zatonsky GV, Shashkov AS, Knirel YA, Jansson PE, et al. Structural studies of the O-specific polysaccharide of *Vibrio cholerae* O8 using solvolysis with triflic acid. Carbohydr Res. 2001;330(1):83-92.
52. Senchenkova SN, Shashkov AS, Knirel YA, Esteve C, Alcaide E, Merino S, et al. Structure of a polysaccharide from the lipopolysaccharide of *Vibrio vulnificus* clinical

isolate YJ016 containing 2-acetimidoylamino-2-deoxy-L-galacturonic acid. Carbohydr Res. 2009;344(8):1009-13.
53. Morrison MJ, Imperiali B. Biosynthesis of UDP-N,N'-diacetylbacillosamine in *Acinetobacter baumannii*: Biochemical characterization and correlation to existing pathways. Arch Biochem Biophys. 2013;536(1):72-80.
54. Hartley MD, Morrison MJ, Aas FE, Borud B, Koomey M, Imperiali B. Biochemical characterization of the O-linked glycosylation pathway in *Neisseria gonorrhoeae* responsible for biosynthesis of protein glycans containing N,N'-diacetylbacillosamine. Biochemistry. 2011;50(22):4936-48.
55. Stimson E, Virji M, Makepeace K, Dell A, Morris HR, Payne G, et al. Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose. Mol Microbiol. 1995;17(6):1201-14.

Tables

Table 1. NMR data for native polysaccharide (PS) and methanolized oligosaccharide (OS, with β-QuiNAc4NAc) of *S. suis* serotype 3 capsular polysaccharide.[1]

Unit		1	2	3	4	5	6
QuiNAc4NAc A	H	4.50	3.82	3.71	3.59	3.59	1.22
PS	C	101.7	55.9	80.3	56.1	71.5	17.7
QuiNAc4NAc A	H	4.45	3.83	3.82	3.62	3.63	1.24
OS	C	102.5	56.4	80.3	56.6	71.8	17.9
GlcA B	H	4.31	3.19	3.51	3.67	3.58	
PS	C	105.0	73.1	74.2	81.6	77.5	
GlcA B	H	4.44	3.25	3.49	3.59	3.99	
OS	C	104.5	73.5	75.8	72.2	75.7	171.6

[1] Bruker Avance III 600 MHz, at 40°C. NAc in PS: 1.92/23.1; 2.00/23.1 ppm. In OS, Me-glycoside 3.49/58.2 ppm; Me ester 3.83/54.0 ppm.

Table 2. Comparison of UDP-QuiNAc4NAc synthesis gene products of *C. jejuni* ATCC 700819, *N. gonorrheae* FA1090, and *A. baumannii* LUH5542 with *cps* gene products of *S. suis* serotypes 3, 13 and 18.

					Similar *cps* gene products of *S. suis* serotypes 3, 13 and 18									
						Amino acids			Amino acids			Amino acids		
Species	Gene	Size	Product (putative)	Function/ Pathway	*cps* gene product	Size	Identity (%)	*cps* gene product	Size	Identity (%)	*cps* gene product	Size	Identity (%)	Similar *cps* gene products of the other serotypes[1]
C. jejuni	*pglF*	590	Dehydrogenase	UDP-4-keto sugar synthesis	Cps3E	608	216/581 (37.2%)	Cps18E	608	212/582 (36.4%)	Cps13E	608	215/582 (36.9%)	Cps4E, 5E, 7E, 9E, 10E, 11E, 12E, 17E, 19E, 21E, 23E, 24E, 26E, 28E, 29E, 30E, 31E, 33E, and chzE share 36-38% amino acid identities with PglF (coverage: 82-96%)
C. jejuni	*pglE*	386	Aminotransferase	UDP-4-amino sugar synthesis	Cps3G	416	157/407 (38.6%)	Cps18 G	416	151/407 (37.1%)	Cps13 G	416	153/407 (37.6%)	
C. jejuni	*pglD*	195	Acetyltransferase	UDT-QuiNAc4NAc sugar synthesis	Cps3I	202	79/202 (39.1%)	Cps18I	210	77/203 (37.9%)	Cps13 N	206	58/201 (28.9%)	

N. gonorrhoeae	*pglD*	636	Dehydrogenase	UDP-4-keto sugar synthesis	Cps3E	608	223/572 (39.0%)	Cps18E	608	227/613 (37.0%)	Cps13E	608	217/562 (38.6%)	Cps4E, 5E, 7E, 9E, 10E, 11E, 12E, 17E, 19E, 21E, 23E, 24E, 26E, 28E, 29E, 30E, 31E, 33E, and chzE share 36-40% amino acid identities with PglD (coverage: 86-96%)
N. gonorrhoeae	*pglC*	391	Aminotransferase	UDP-4-amino sugar synthesis	Cps3G	416	83/245 (33.9%)	Cps18G	416	81/247 (32.8%)	Cps13G	416	82/247 (33.2%)	
N. gonorrhoeae	*pglB*	413	Acetyltransferase and glycosyltransferase	UDT-QuiNAc4NAc sugar synthesis N-terminal (1st-200th, contains sugar transferase domain)	Cps3H	202	115/199 (57.8%)	Cps18H	210	115/199 (57.8%)	Cps13H	206	94/202 (46.5%)	Cps1E, 1/2E, 2E, 4F, 5F, 6E, 7F, 8E, 9F, 10F, 11F, 12F, 14E, 15E, 16E, 17G, 19G, 21G, 22E, 23F, 24G, 25E, 26H, 27E, 28F, 29G, 30F, 31G, 32E, 33G, 34E, and chzG share 30-44% amino acid

														identities with N-terminal of PglB (coverage: 71-97%)
				C-terminal (201st-413th,contains acetyltransferase domain)	Cps3I	202	83/204 (40.7%)	Cps18I	210	81/205 (39.5%)	Cps13N	206	60/204 (29.4%)	
A. baumannii	*weeK*	605	Dehydrogenase	UDP-4-keto sugar synthesis	Cps3E	608	211/519 (40.7%)	Cps18E	608	214/529 (40.5%)	Cps13E	608	213/529 (40.3%)	Cps4E, 5E, 7E, 9E, 10E, 11E, 12E, 17E, 19E, 21E, 23E, 24E, 26E, 28E, 29E, 30E, 31E, 33E, and chzE share 38-46% amino acid identities with WeeK (coverage: 66-90%)
A. baumannii	*weeJ*	391	Aminotransferase	UDP-4-amino sugar synthesis	Cps3G	416	86/309 (27.8%)	Cps18G	416	74/255 (29.0%)	Cps13G	416	78/267 (29.2%)	Cps4G, 5G, 7G, 17H, 19H, and 23G share 30-31% amino acid

															identities with WeeJ (coverage:97-100%)
A. baumannii	*weeI*	216	Acetyltransferase	UDT-QuiNAc4NAc sugar synthesis	Cps3I	202	64/217 (29.5%)	Cps18I	210	62/212 (29.2%)	Cps13N	206	34/113 (30.1%)		

[1] *cps* gene products of the other serotypes, which shared coverage >60% and identities >30% with UDP-QuiNAc4NAc synthesis gene products, are shown.

Table 3. Comparison of Cps3E, 3G, and 3I of *S. suis* serotype 3 reference strain with *cps* gene products of other *S. suis* serotype reference strains.

				Similar *cps* gene products of *S. suis*						
					Amino acids			Amino acids		
Gene	Size	Product (putative)	Putative Function/Pathway	*cps* gene product	Size	Identity (%)	*cps* gene product	Size	Identity (%)	*cps* gene products of the other serotypes [1]
cps3E	608	Dehydrogenase	UDP-4-keto sugar synthesis	Cps18E	608	583/608 (95.9%)	Cps13E	608	585/608 (96.2%)	Cps4E, 5E, 7E, 9E, 10E, 11E, 12E, 17E, 19E, 21E, 23E, 24E, 26E, 28E, 29E, 30E, 31E, 33E, and chzE share 76-99% amino acid identities with Cps3E (coverage: 99-100%)
cps3G	416	Aminotransferase	UDP-4-amino sugar synthesis	Cps18G	416	382/416 (91.8%)	Cps13G	416	400/416 (96.2%)	
cps3I	202	Acetyltransferase	UDP-diNAcBac sugar synthesis	Cps18I	210	121/200 (60.5%)	Cps13N	206	68/207 (32.9%)	

[1] *cps* gene products of the other serotypes, which shared coverage >60% and identities >30% with *cps*3 gene products, are shown.

Table 4. NMR data for native (PS), O-deacylated (DPS) and O-deacylated oxidized (DPS-ox) polysaccharides of *S. suis* serotype 18 capsular polysaccharide.[1]

Unit		1	2	3	4	5	6
α-GalNAc A	H	5.22	4.47	4.03	4.04	4.31	3.76; 3.80
PS	C	93.7	48.6	76.0	67.5	72.1	62.6
α-GalNAc A	H	5.19	4.51	4.07	4.09	4.27	3.76; 3.80
DPS	C	93.3	48.7	76.1	67.5	72.4	62.6
α-GalNAc A	H	5.12	4.44	4.02	4.14	4.17	3.77; 3.77
DPS-ox	C	95.4	49.0	75.8	66.9	71.8	62.2
α-GalNAc B	H	5.18	4.30	3.82	4.31	3.89	3.80; 3.80
PS	C	98.0	49.2	78.0	69.7	71.7	61.8
α-GalNAc B	H	5.18	4.30	3.82	4.36	3.89	3.80; 3.80
DPS	C	98.0	49.2	78.3	69.3	71.7	61.8
α-GalNAc B	H	5.16	4.34	3.78	4.36	3.88	3.80; 3.80
DPS-ox	C	98.3	49.1	79.2	69.0	71.6	61.8
α-QuiNAc4NAc D	H	4.97	4.16	3.89	3.83	3.69	1.18
PS	C	96.6	53.7	72.9	58.1	69.1	17.9
α-QuiNAc4NAc D	H	4.97	4.16	3.89	3.83	3.69	1.18
DPS	C	96.6	53.7	72.9	58.1	69.1	17.9
α-QuiNAc4NAc D	H	5.00	4.14	3.91	3.84	3.71	1.18
DPS-ox	C	96.3	53.8	73.3	58.1	69.0	17.6
β-Glc E	H	4.66	3.23	3.42	3.32	3.42	3.66; 3.88
PS	C	103.4	74.5	77.4	71.3	77.4	62.6
β-Glc E	H	4.71	3.23	3.46	3.32	3.40	3.66; 3.88
DPS	C	103.4	74.5	77.4	71.3	77.4	62.6
β-GalA F	H	4.73	4.09	4.18	5.78	4.46	
PS	C	103.4	74.6	74.0	68.4	73.7	
β-GalA F	H	4.63	4.08	4.02	4.53	4.19	
DPS	C	103.3	74.3	76.2	66.1	76.0	

β-GalA F	H	4.47	3.61	3.78	4.38	4.00
DPS-ox	C	105.4	70.0	78.3	67.8	76.4

[1] Bruker Avance III 600 MHz, at 40°C. NAc in PS and DPS: 1.96/23.3; 2.02/24.1; 2.06/23.5 ppm. OAc in PS: 2.16/21.5 ppm.

Table 5. Putative function of *cps* gene products of *S. suis* serotype 3 reference strain involved in the synthesis of its capsular polysaccharide.

Gene	Conserved domain	Product (putative)	Putative Function/Pathway	Description
cps3A	pfam03816: LytR_cpsA_psr; Cell envelope-related transcriptional attenuator domain pfam02916: DNA_PPF; DNA polymerase processivity factor	Integral membrane regulatory protein Wzg	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps3B	cl26080: GNVR super family; G-rich domain on putative tyrosine kinase	Chain length determinant protein/polysaccharide export protein Wzd	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps3C	TIGR01007: eps_fam; capsular exopolysaccharide family	Tyrosine-protein kinase Wze	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps3D	cl23724: PHP super family; Polymerase and Histidinol Phosphatase domain	Protein-tyrosine phosphatase Wzh	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*

cps3E	COG1086: FlaA1; NDP-sugar epimerase	Nucleoside-diphosphate sugar epimerase	UDP-QuiNAc4NAc sugar synthesis	Table 2
cps3F	COG3177: COG3177; Fic family protein	Fic/DOC family protein	Unknown	
cps3G	cl18945: AAT_I super family; Aspartate aminotransferase superfamily (fold type I) of pyridoxal phosphate - dependent enzymes	Aminotransferase	UDP-QuiNAc4NAc sugar synthesis	Table 2
cps3H	pfam02397: Bac_transf; Bacterial sugar transferase	Initial glycosyltransferase	Addtion of QuiNAc4NAc to the lipid carrier	Similar to undecaprenyl phosphate N,'N'-diacetylbacillosamine 1-phosphate transferase PglC of *C. jejuni* ATCC 700819 (amino acid identity, 60.8%; coverage, 99.0%) Almost identical to Cps18H (amino acid identity, 96.6%; coverage, 100%)
cps3I	TIGR03570: NeuD_NnaD; sugar O-acyltransferase, sialic acid O-acetyltransferase NeuD	Sugar acetyltransferase	UDP-QuiNAc4NAc sugar synthesis	Table 2

	family			
cps3J	cl11394: Glyco_tranf_GTA_type super family; Glycosyltransferase family A (GT-A)	Glycosyltransferase	Addition of GlcA to QuiNAc4NAc ?	*cps3* gene cluster specific
cps3K	cl25977: RfbX super family; membrane protein involved in the export of O-antigen and teichoic acid	Flippase Wzx	Transportation of repeat units	*cps3* gene cluster specific
cps3L	pfam04932: Wzy_C; O-Antigen ligase	Oligosaccharide repeat unit polymerase Wzy	Polymerization of repeat units	*cps3* gene cluster specific
cps3M	No conserved domain	Hypothetical protein	Unknown	
cps3N	cl27617: UDPG_MGDP_dh super family; UDP-glucose/GDP-mannose dehydrogenase family, central domain smart00530: HTH_XRE; Helix-turn-helix XRE-	UDP-glucose dehydrogenase	UDP-GlcA sugar synthesis	Similar to UDP-glucose 6-dehydrogenase Ugd of *Streptococcus pneumoniae* (amino acid identity, 40-80%; coverage, 99%)

	family like protein		
cps3O	pfam13191: AAA_16; AAA ATPase domain	Hypothetical protein (probable pseudogene)	Unknown
cps3P		Hypothetical protein (probable pseudogene)	Unknown

Table 6. Putative function of *cps* gene products of *S. suis* serotype 18 reference strain involved in the synthesis of its capsular polysaccharide.

Gene	Conserved domain	Product (putative)	Putative Function/Pathway	Description
cps18A	pfam03816: LytR_cpsA_psr; Cell envelope-related transcriptional attenuator domain pfam02916: DNA_PPF; DNA polymerase processivity factor	Integral membrane regulatory protein Wzg	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps18B	cl26080: GNVR super family; G-rich domain on putative tyrosine kinase	Chain length determinant protein/polysaccharide export protein Wzd	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps18C	TIGR01007: eps_fam; capsular exopolysaccharide family	Tyrosine-protein kinase Wze	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps18D	cl23724: PHP super family; Polymerase and Histidinol Phosphatase domain	Protein-tyrosine phosphatase Wzh	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps18E	COG1086: FlaA1; NDP-sugar epimerase	Nucleoside-diphosphate sugar epimerase	UDP-QuiNAc4NAc sugar synthesis	Table 2

cps18F	COG3177: COG3177; Fic family protein	Fic/DOC family protein	Unknown	
cps18G	cl18945: AAT_I super family; Aspartate aminotransferase superfamily (fold type I) of pyridoxal phosphate - dependent enzyme	Aminotransferase	UDP-QuiNAc4NAc sugar synthesis	Table 2
cps18H	pfam02397: Bac_transf; Bacterial sugar transferase	Initial glycosyltransferase	Addition of QuiNAc4NAc to the lipid carrier	Similar to undecaprenyl phosphate N,'N'-diacetylbacillosamine 1-phosphate transferase PglC of *C. jejuni* ATCC 700819 (amino acid identity, 61.3%; coverage, 99.0%). Almost identical to Cps3H (amino acid identity, 96.6%; coverage, 100%).
cps18I	TIGR03570: NeuD_NnaD; sugar O-acyltransferase, sialic acid O-acetyltransferase NeuD family	Sugar acetyltransferase	UDP-QuiNAc4NAc sugar synthesis	Table 2

cps18J	cd03808: GT1_cap1E_like; GT1 family of glycosyltransferase	Glycosyltransferase	Addition of GalNAc to QuiNAc4NAc	Similar to N,'N'-diacetylbacillosaminyl-diphospho-undecaprenol alpha-1,3-N acetylgalactosaminyltransferase PglA of *C. jejuni* ATCC 700819, but the similarity bewtween two proteins is very low (amino acid identity, 26.4%; coverage, 91.2%). *cps18* gene cluster specific
cps18K	cl11394: Glyco_tranf_GTA_type super family; Glycosyltransferase family A (GT-A)	Glycosyltransferase	Unknown (addition of the single branching Glc to GalA4-OAc?)	Partially similar to a region of GalNAc(5)-diNAcBac-PP-undecaprenol beta-1,3-glucosyltransferas PglI of *C. jejuni* ATCC 700819 (amino acid identity, 34.8%; coverage, 29.4%). *cps18* gene cluster specific
cps18L	cl10013: Glycosyltransferase_GTB_type super family; Glycosyltransferase	Glycosyltransferase	Unknown (addition of GalNAc to GalA4-OAc?)	Partially similar to a region of N,'N'-diacetylbacillosaminyl-diphospho-undecaprenol alpha-1,3-N acetylgalactosaminyltransferase PglA of *C. jejuni* ATCC 700819, but the similarity bewtween two proteins is very low (amino acid identity, 28.7%;

				coverage, 25.0%). *cps18* gene cluster specific
cps18M	No conserved domain	Glycosyltransferase ?	Unknown (addition of GalA4-OAc to GalNAc?)	Similar to putative glycosyl transferases WcyD of *S. pneumoniae* (amino acid identity, 40-41%; coverage, 96-99%). Function of WcyD is unknown. *cps18* gene cluster specific
cps18N	No conserved domain	Oligosaccharide repeat unit polymerase Wzy?	Polymerization of repeat units?	Similar to the putative oligosaccharide repeat unit polymerase of *Brochothrix thermosphacta*, but the similarity bewtween two proteins is very low (amino acid identity, 22.6%; coverage, 80-84%). *cps18* gene cluster specific
cps18O	cl09326: MATE_like super family; integral membrane proteins involved in exporting metabolites across the cell membrane	Flippase Wzx	Transportation of repeat units	Similar to Flippase Wzx of *S. pneumoniae* tp 25/38, sp 65/81 (amino acid identity, 40.8%; coverage, 91.5%). *cps18* gene cluster specific

cps18P	pfam08942: DUF1919 super family; unknown function COG3955: COG3955; Uncharacterized DUF1919 family protein (Cell wall/membrane/envelope biogenesis)	Sugar acetyltransferase ?	Unknown (GalA4-OAc synthesis?)	Similar to putative acetyl transferase WcwC of *Streptococcus pneumoniae* (amino acid identity, 35-42%; coverage, 93-98%). *cps18* gene cluster specific
cps18Q	cl25409: SDR super family; Short-chain dehydrogenases/reductase	Epimerase	UDP-GalA sugar synthesis	Similar to putative epimerase Gla of *Streptococcus pneumoniae* (amino acid identity, 70%; coverage, 100%).
cps18R	cl27617: UDPG_MGDP_dh super family; UDP-glucose/GDP-mannose dehydrogenase family, central domain smart00530: HTH_XRE; Helix-turn-helix XRE-family like protein	UDP-glucose dehydrogenase	UDP-GalA sugar synthesis	Similar to UDP-glucose 6-dehydrogenase Ugd of *Streptococcus pneumoniae* (amino acid identity, 40-80%; coverage, 99%).
cps18S	cl18945: AAT_I super family; Aspartate aminotransferase superfamily (fold type I) of	Serine hydroxymethyltransferase	Unknown	Similar to serine hydroxymethyltransferase of *Streptococcus pneumoniae* (amino acid

	pyridoxal phosphate - dependent enzyme			identity, 55-74%; coverage, 83-84%).
cps18T	pfam13424: TPR_12; Tetratricopeptide repeat	Hypothetical protein	Unknown	
cps18U	pfam13191: AAA_16; AAA ATPase domain	Hypothetical protein (probable pseudogene)	Unknown	
cps18V		Hypothetical protein (probable pseudogene)	Unknown	

Table S1. Other reported di-*N*-acetyl-bacillosamine (QuiNAc4NAc)-containing polysaccharides and oligosaccharides from prokaryotes.

Organism (Strain)	Structure	Reference
Capsular polysaccharides		
Alteromonas sp. (CMM 155)	[→3)-α-D-Gal*p*NAc-(1→4)-α-L-GalA*p*NAc-(1→3)-α-D-Qui*p*NAc4NAc-(1→3)-β-D-Qui*p*4NAlaAc-(1→]$_n$	(33)
Colwellia psychrerythraea (34H)	[→4)-β-D-Glc*p*NAcA-(1→3)-β-D-Qui*p*NAc4NAc-(1→3)-β-D-Gal*p*NAc-(1→]$_n$	(34)
Lipopolysaccharides (O-antigens)		
Acinetobacter haemolyticus (ATCC 17906)	[→4)-α-D-Gal*p*NAcA-(1→3)-β-D-Qui*p*NAc4NAc-(1→4)-α-D-Gal*p*NAcA-(1→]$_n$ | 6 D-Ala	(36)
Flavobacterium psychrophilum (259-93)	[→4)-α-L-Fuc*p*NAc-(1→3)-α-D-Qui*p*NAc4NAcyl-(1→2)- α-L-Rha*p*-(1→]$_n$ Where Acyl is (*3S*,*5S*)-dihydroxylhexanoic acid	(37)
Francisella novicida (U112)	…-α-GalA*p*NAc-(1→4)- α-GalA*p*NAc-(1→3)-β-Qui*p*NAc4NAc-(1→4)-β-Man → Core * Description of an O-chain linked to the LPS core.	(38)
Fusobacterium	[→4)-α-D-Glc*p*-(1→3)-α-L-Fuc*p*Am-(1→3)-β-D-Qui*p*NAc4N-(1→Acid-P→]$_n$	(39)

necrophorum (LA 81.617)	| (*S*)-3-hydroxybutanoic acid Where Acid is 2-amino-2-methyl-3,4,5-trihydroxypentanoic acid.	
Pragia fontium (97U124)	[→3)-α-L-Fuc*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Qui*p*NAc4N-(1→]$_n$ | D-Glyceroyl	(40)
Pseudomonas aeruginosa IID 1001 (ATCC 27577)	[→6)-α-D-GlcNAc-(1→4)-α-L-GalNAcA-(1→3)-β-D-QuiN4NAc-(1→2)-α-L-Rha-(1→]$_n$ | | | 80% O-Ac 3-Hydroxybutanoic acid 20% O-Ac	(41)
Pseudomonas aeruginosa Habs O3	[→6)-α-D-GlcNAc-(1→4)-α-L-GalNAcA-(1→3)-β-D-QuiNAc4N-(1→2)-α-L-Rha-(1→]$_n$ | 3 | | 3 O-Ac 3-Hydroxybutanoic acid 40% O-Ac	(42)
Pseudomonas aeruginosa Lanyi O1	[→6)-α-D-GlcNAc-(1→4)-α-L-GalNAcA-(1→3)-β-D-QuiNAc4N-(1→2)-α-L-Rha-(1→]$_n$ | | 3 3-Hydroxybutanoic acid 40% O-Ac	(42)
Pseudomonas aeruginosa Wokatsch O13	[→6)-α-D-GlcNAc-(1→4)-α-L-GalNAcA-(1→3)-β-D-QuiNAc4NAc-(1→3)-α-L-Rha-(1→]$_n$	(42)
Pseudoalteromonas haloplanktis	[→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Qui*p*NHb4NHb-(1→2)-α-L-Ido*p*A-(1→]$_n$ | 4	(43)

KMM 223 (44-1)	α-D-Qui*p*NAc4NAc Where ‘’b’’ in NHb is (S)-3-hydroxybutyramide	
Pseudomonas aurantiaca (IMV 31)	[→3)-β-D-QuiNAc4NAc-(1→3)-α-L-FucNAc-(1→3)-α-L-FucNAc-(1→]$_n$	(44, 45)
Pseudomonas reactans	[→3)-β-D-Qui*p*NAlaAc4NAlaAc-(1→3)-α-D-Glc*p*2Am-(1→3)-α-D-Qui*p*NAc4NAc-(1→]$_n$	(46)
Rheinheimera pacifica (KMM 1406)	[→4)-α-D-Gal*p*NAc-(1→4)-α-L-Gal*p*NAcA-(1→3)-β-D-Qui*p*NAc4NAc-(1→2)-β-D-Qui*p*4NDAlaAc-(1→4)-α-D-Gal*p*NAcA-(1→]$_n$	(47)
Taylorella asinigenitalis (ATCC 700933)	[→3)-β-D-Qui*p*NAc4NAc-(1→3)-β-D-Glc*p*NAmA-(1→]$_n$	(48)
Vibrio cholerae O:3	[→2)-α-D-Hep*p*-(1→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Qui*p*NAc4NAcyl-(1→]$_n$ | 3 α-L-Asc*p* Where Acyl is 3,5-dihydroxylhexanoic acid	(49)
Vibrio cholerae O:5	[→3)-β-D-Qui*p*NAc4NAc-(1→4)-β-D-Man*p*NAcA-(1→]$_n$ | 3 α-D-Fuc*p*3NX	(50)

Vibrio cholerae O8	[→4)-β-D-Glc*p*NAc3NAcylAN-(1→4)-β-D-Man*p*NAc3NAcAN-(1→4)-α-L-Gul*p*NAc3NAcA-(1→3)-β-D-Qui*p*NAc4NAc-(1→]$_n$	(51)
Vibrio vulnificus (YJ016)	[→3)-α-L-Gal*p*NAmA-(1→3)-β-D-Qui*p*NAc4NAc-(1→3)-α-L-Fuc*p*-(1→3)-α-D-Glc*p*NAc-(1→]$_n$ | 4 β-D-Glc*p*NAc--6Ac (70 %)	(52)
Glycoproteins		
Acinetobacter baumannii	*O*-linked glycosylation	(53)
Campylobacter jejuni	*N*-linked glycosylation	(24)
Neisseria spp.	*O*-linked glycosylation	(54, 55)

Figures

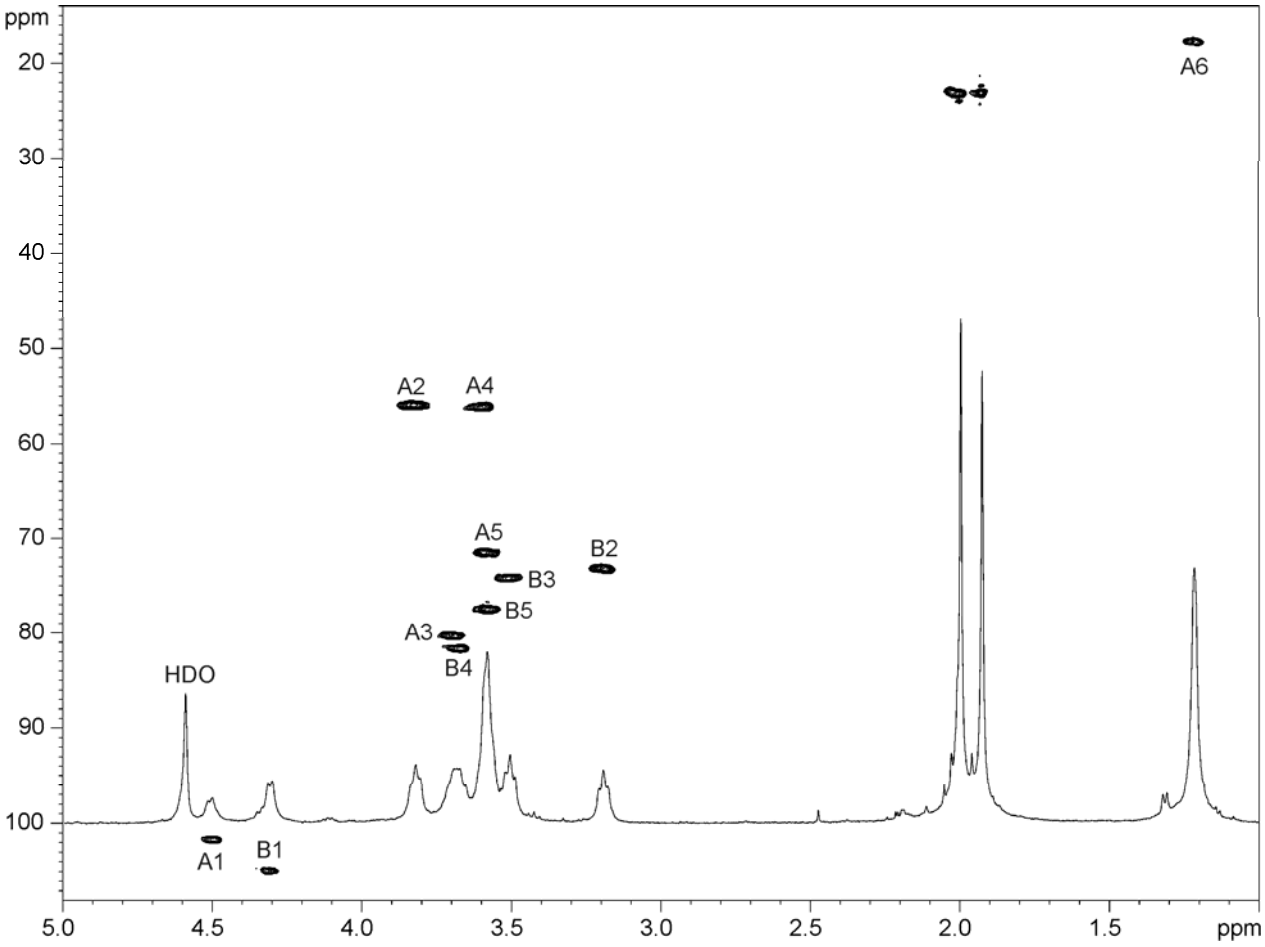


Figure 1. ^{1}H-^{13}C HSQC spectrum of the *S. suis* serotype 3 capsular polysaccharide.

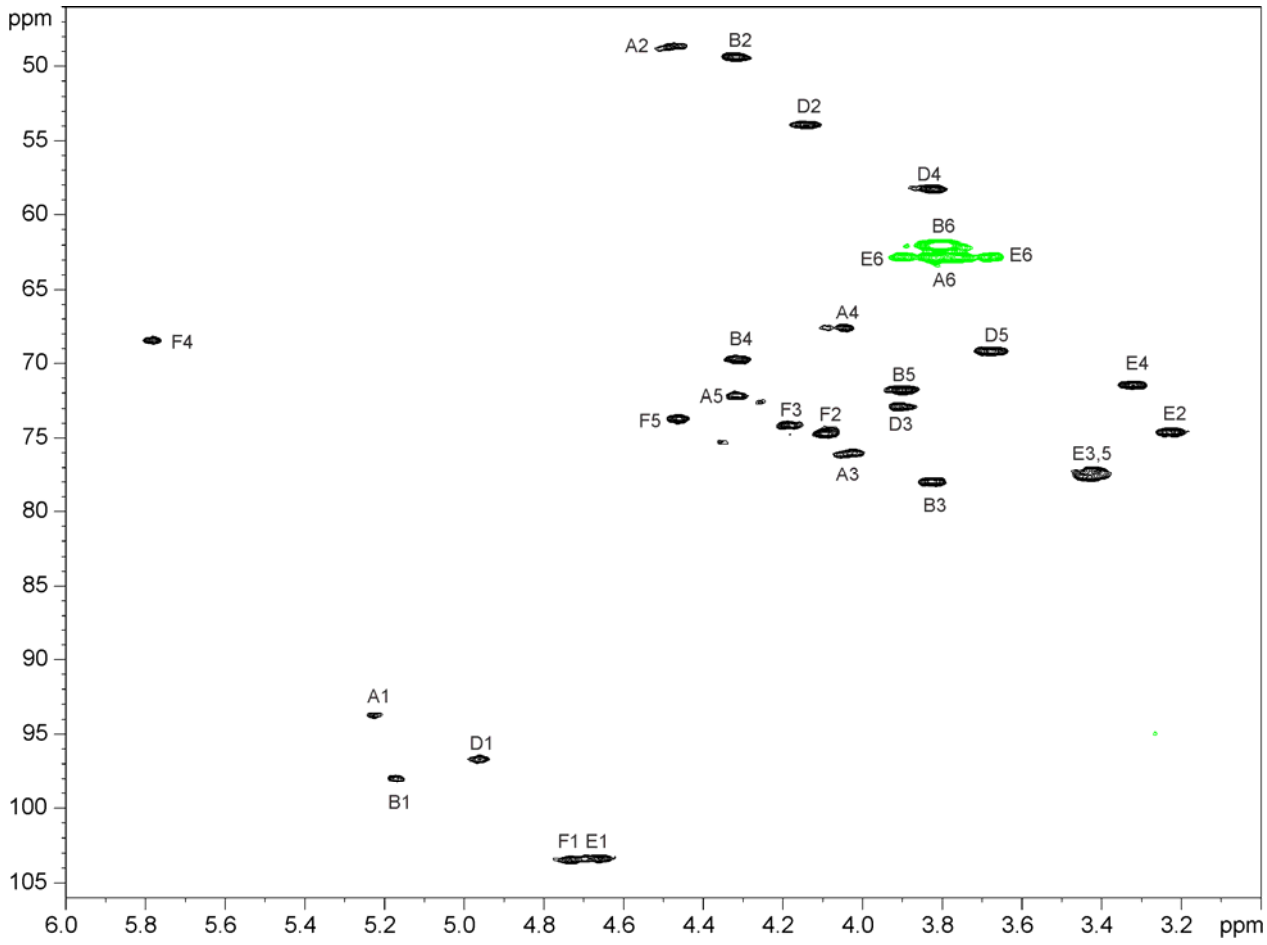


Figure 2. ^{1}H-^{13}C HSQC spectrum of the *S. suis* serotype 18 capsular polysaccharide.

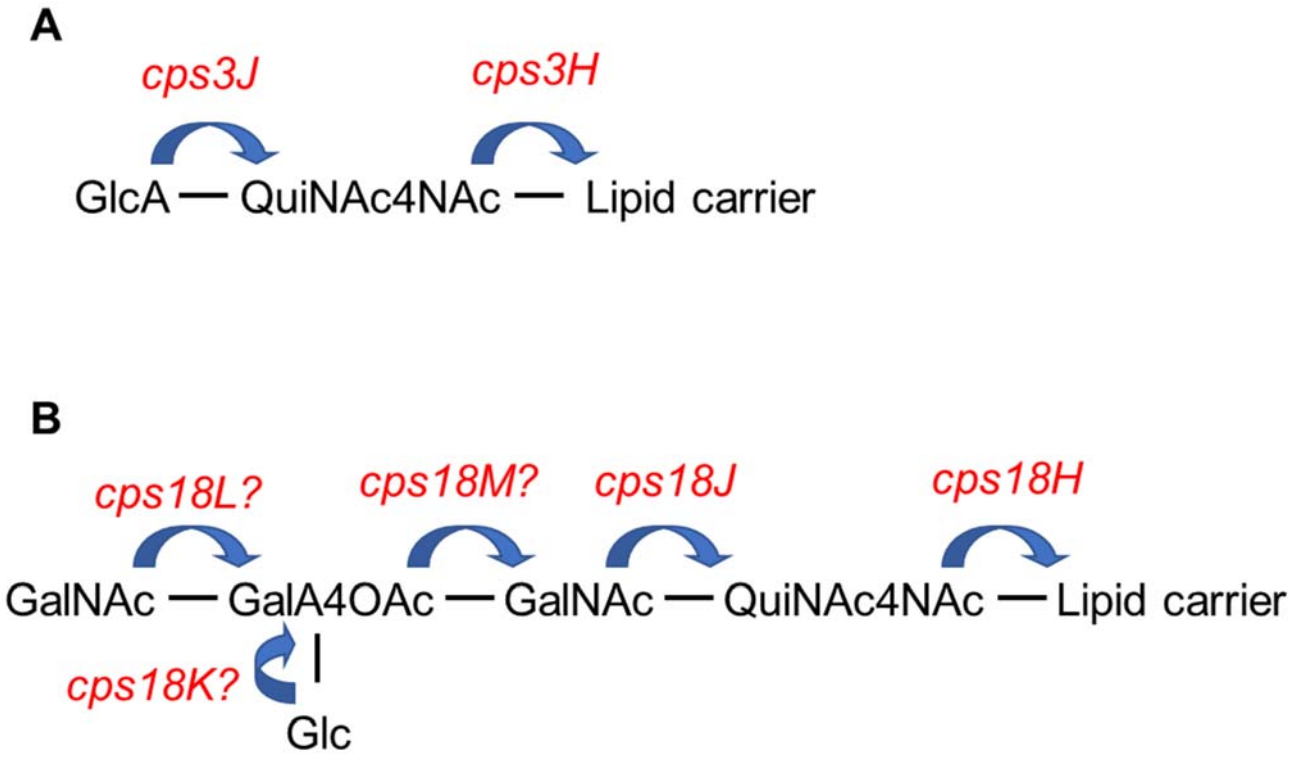


Figure 3. Tentative correlation between structure and genes encoding enzymes responsible for the biosynthesis of *S. suis* serotypes 3 (A) and 18 (B) capsular polysaccharides (CPS).

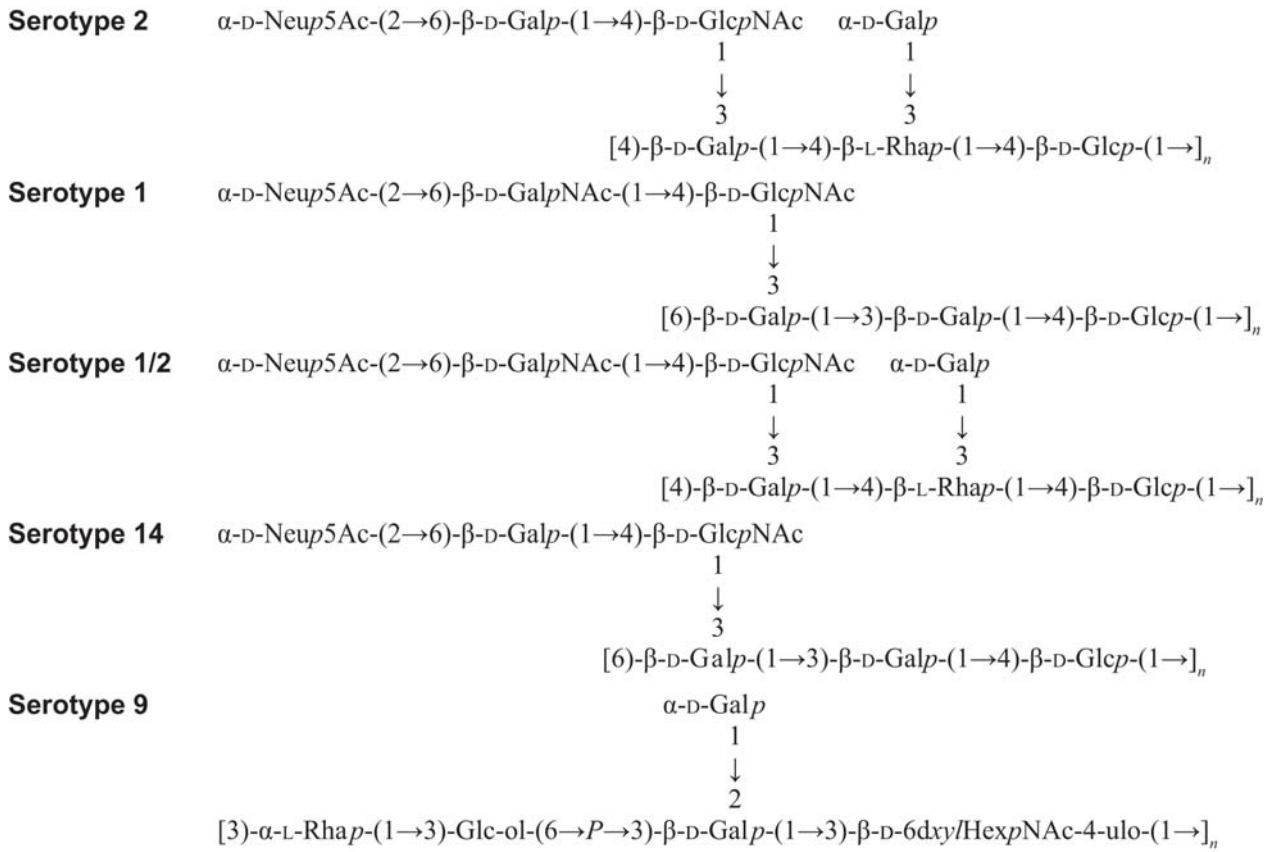


Figure S1. Reported structures for the known capsular polysaccharide repeating units of *S. suis* serotypes 1 (14), 1/2 (14), 2 (12), 14 (13), and 9 (15).

ARTICLE III

Structure determination of *Streptococcus suis* serotypes 7 and 8 capsular polysaccharides and assignment of functions of the *cps* locus genes involved in their biosynthesis

---

Guillaume Goyette-Desjardins [1,2], Evgeny Vinogradov [2,3], Masatoshi Okura [4], Daisuke Takamatsu [4,5], Marcelo Gottschalk [1,2], and Mariela Segura [1,2]

[1] Swine and Poultry Infectious Diseases Research Center, Faculty of Veterinary Medicine, University of Montreal, 3200 Sicotte St., St-Hyacinthe, Quebec, Canada, J2S 2M2

[2] Canadian Glycomics Network (GlycoNet), University of Alberta, 11227 Saskatchewan Dr., Edmonton, Alberta, Canada, T6G 2G2

[3] National Research Council, 100 Sussex Dr., Ottawa, Ontario, Canada, K1A 0R6

[4] Division of Bacterial and Parasitic Disease, National Institute of Animal Health, National Agriculture and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

[5] The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1193, Japan

Publié dans *Carbohydrate Research.* **2019**. 473:36-45.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai participé activement à la conception de l'étude, aux expériences (production et purification des polysaccharides, analyses sérologiques; 40%), à l'analyse des résultats et à l'écriture de l'article.

Abstract

Streptococcus suis serotypes 7 and 8 are counted among the top six *S. suis* serotypes causing clinical disease in pigs. Yet, limited information is available on these serotypes. Since *S. suis* serotyping system is based upon capsular polysaccharide (CPS) antigenicity and the CPS is considered a major virulence factor for encapsulated pathogens, here we determined for the first time the chemical compositions and structures of serotypes 7 and 8 CPSs. Chemical and spectroscopic data gave the following repeating unit sequences: [3)L-Rha(α1-*P*-2)D-Gal(α1-4)D-GlcA(β1-3)D-FucNAc4N(α1-]$_n$ for serotype 7 and [2)L-Rha(α1-*P*-4)D-ManNAc(β1-4)D-Glc(α1-]$_n$ for serotype 8. As serotype 8 CPS is identical to *Streptococcus pneumoniae* type 19F CPS, dot-blot analyses showed a strong reaction of the 19F polysaccharide with reference anti-*S. suis* serotype 8 rabbit serum. A correlation between *S. suis* serotypes 7 and 8 sequences and genes of those serotypes' *loci* encoding putative glycosyltransferases and polymerases responsible for the biosynthesis of the repeating units was tentatively established. Knowledge of CPS structure and composition will contribute to better dissect the role of this bacterial component in the pathogenesis of the disease caused by *S. suis* serotypes 7 and 8.

1. Introduction

Streptococcus suis is an encapsulated Gram-positive bacterium and one of the most important causes of bacterial infection and death in post-weaned piglets. This infection causes important economic losses to the swine industry and raise concerns about animal welfare. Striking manifestations of the disease in pigs are septicemia and meningitis, but other clinical manifestations can also be present as endocarditis and arthritis. Pigs may acquire *S. suis* from the sows and through piglet-to-piglet transmission. *S. suis* is mainly localized in the upper respiratory tract of pigs, more particularly the tonsils and nasal cavities. Almost 100% of pig farms worldwide have carrier animals, although only a limited number of pigs develop clinical disease in the presence of preventive medication (1). Yet, recent implementation of compulsory reduction in the use of antibiotics in livestock production has led to a worriedly increase of *S. suis* clinical cases in swine productions. *S. suis* is also an emerging zoonotic agent, being responsible for septic shock, meningitis and other less common infections usually related to generalized septicemia. This zoonosis is of particular importance worldwide to people in close contact with infected pigs and/or pork-derived products. Though human infections in Western countries are considered an occupational disease (affecting mainly workers in the swine/pork industries), in some Asian countries, the general population is at risk since many cases of infection have been reported after ingestion of contaminated raw pork products (1, 2).

Cell-associated capsular polysaccharide (CPS) is considered to be one of *S. suis* most important virulence factors (3, 4). In fact, the originally described 35 serotypes of *S. suis* are based upon CPS antigenicity. Globally, the top 10 predominant serotypes in *S. suis* isolates reported between 2002 to 2013 from clinical cases in pigs are, in decreasing order, serotypes 2, 9, 3, 1/2, 8, 7, 4, 22, 5, and 1. However, there is a clear geographical effect on the distribution of serotypes (2). Studies from Asia and South America report mainly serotype 2 (44.2% and 57.6% of all cases, respectively), while serotype 9 predominates in Europe with 61.0% of infections in swine. In North America, there is no clear dominating serotype as the top distribution is as follows: serotype 2 (24.3%), 3 (21.0%), 1/2 (13.0%), 8 (10.5%), and 7 (9.0%). Despite not being the most prevalent, infections caused by serotypes 7 and 8 in swine

remain important, as they are always found among the top six serotypes per continent: 8.0% for serotype 7 and 1.6% for serotype 8 in South America; 3.3% for both serotypes 7 and 8 in Asia; and 6.7% for serotype 7 and 4.8% for serotype 8 in Europe (2).

However, since the publication of that review, other epidemiological reports were published. In North America, a recent study on *S. suis* serotype distribution from diseased pigs in the province of Québec, Canada, over 2012-2014 reported a prevalence of 6% and 5% for serotypes 7 and 8, respectively (5). As for the situation in Asia, a study on *S. suis* serotype distribution among isolates from pig carcasses sold in wet markets in Chiang Mai Province, northern Thailand, reported that serotypes 7 and 8 both ranked 5th in importance, with 2.5% of isolates each (6). Other epidemiological studies in Thailand did not associate serotypes 7 or 8 to diseased pigs, but these serotypes were found at relatively high prevalence in nasal cavities and/or tonsils of healthy pigs (7, 8). Another study throughout Korea reported serotypes 7 and 8 incidence of 3.5% and 1.4%, respectively, in slaughterhouse carcasses; while their occurrence in diseased pigs was higher (4.1% and 5.1%, respectively) (9). Although serotypes 7 and 8 have so far not been isolated from human patients, an eventual role in zoonosis cannot be completely ruled out, especially when considered the popularity of local raw pork dishes in South-East Asia (2, 10). Additionally, two recent field studies on Austrian farms with recurrent infections caused by *S. suis* serotype 7 investigated the use of antibiotics and/or of vaccination with a bacterin (killed whole bacteria) in order to better prevent infections with this serotype (11, 12).

Compared to the extensively studied serotype 2, the molecular aspects of the pathogenesis of the disease caused by serotypes 7 and 8 and the involved virulence factors are largely unknown (3). In fact, knowledge on the contribution of CPS structural diversity to *S. suis* pathogenesis, from colonization to infection of its host, is required in order to help devise new control strategies against *S. suis* infections. So far, our team has been the first, and the only one, to report and expand the known CPS structures of epidemiologically relevant serotypes of *S. suis*. Since 2010, we have published complete structural determination for the CPSs of *S. suis* serotype 2 (13), serotype 14 (14), serotypes 1 & 1/2 (15), serotype 9 (16) and serotypes 3 & 18 (17). In this work, we describe for the first time the primary structures of the

CPS polymer for the reference strains of *S. suis* serotypes 7 and 8 and tentatively assign the functions of the *cps* locus genes involved in their biosynthesis.

2. Material and Methods

2.1. Capsular polysaccharide production, purification and quality controls

S. suis serotype 7 field strain #1750775 and serotype 8 field strain #1719887 (both isolated from diseased pigs in Quebec, Canada – our collection) were used in this study. For the CPS production, 6 L of fresh Todd-Hewitt Broth (THB; Oxoid, Thermo Fisher Scientific, Nepean, Canada) were inoculated with 2 x 75-mL overnight cultures of the strain in THB, then grown overnight as previously described (16, 17). Bacterial cells from the 6-L culture were pelleted by centrifugation at 10,000*g* for 40 min, and suspended by repeated pipetting in deionized water (ddH_2O). The bacteria were killed by heating at 60 °C for 45 min, which has been confirmed by the absence of growth on blood agar plates. Finally, the content of the tubes was lyophilized for 72 h.

For serotype 7 CPS: cells were boiled in 2% AcOH in water with stirring (3 g dry cells in 100 mL) for 5 min, then cell debris were removed by ultracentrifugation at 110,000 g for 2 h. The solution was dried, then separated on a Biogel P10 column (2.5x60 cm) in 1% AcOH, where the elution was monitored using a refractive index detector (Gilson, Middleton, WI). The CPS was partially hydrolyzed, but significant amount of polymeric fraction was collected, as well as repeating unit, its dimer and trimer.

For serotype 8 CPS: cells were stirred in water (3 g dry cells in 100 mL of water) at room temperature overnight, then cell debris were removed by centrifugation at 30,000 g for 20 min. The solution was dialyzed and dried. The crude CPS was dissolved in 2 mL of water, centrifugated (20,000 g for 10 min) to remove insoluble material, before separation on a Biogel P10 column as described above. The polymeric fraction was collected.

Quality controls for presence of contaminating nucleic acids and proteins were performed as previously described (13-17). The calculated limit of detection ($P \leq 0.05$) for the modified Lowry protein assay was 7 μg/mL.

2.2. NMR spectroscopy

NMR experiments were carried out on a Bruker AVANCE III 600 MHz (^{1}H) spectrometer with 5 mm Z-gradient probe with acetone internal reference (2.225 ppm for ^{1}H and 31.45 ppm for ^{13}C) using standard pulse sequences cosygpprqf (gCOSY), mlevphpr (TOCSY) (mixing time 120 ms), roesyphpr (ROESY) (mixing time 500 ms), hsqcedetgp (HSQC), and hmbcgplpndqf (HMBC) (100 ms long range transfer delay). Resolution was kept < 3 Hz/pt in F2 in H-H correlations and < 5 Hz/pt in F2 of H-C correlations. Number of points in F2 set so to have AQ about 0.8 s for H-H correlations, 0.24 s for C-H HSQC. Number of points in F1 was 1/4 of F2. The spectra were processed and analyzed using the Bruker Topspin 2.1 program.

2.3. Determination of neutral and amino sugars as alditol acetates

Monosaccharides were detected as reduced and acetylated derivatives (alditol acetates). Polysaccharide sample (0.2 mg) with inositol internal standard were hydrolyzed with 3 M trifluoroacetic acid (120 °C, 3 h). The sample was then dried, reduced with $NaBD_4$, and the excess of reagent destroyed with 0.5 mL of AcOH. The obtained solution was dried under an air stream, dried twice with addition of MeOH (1 mL), acetylated with 0.4 mL Ac_2O-0.4 mL pyridine for 30 min at 100 °C, dried, and finally analyzed by Gas-Liquid Chromatography-Mass Spectrometry (GLC-MS) (Thermo ion-trap instrument, capillary column DB-17, 160-260°C by 4°/min).

2.4. Absolute configurations

For the determination of absolute configuration, to the hydrolyzed polysaccharides (0.3 mg; as described above) were added either (R)- or racemic 2-octanol (0.2 mL) and acetyl chloride (20 μL) at room temperature, heated at 100 °C for 2 h, dried by air stream, acetylated (0.2 mL Ac_2O-0.2 mL pyridine, 100 °C, 30 min), dried, and finally analyzed by GLC-MS as described above.

2.5. Dot-blot

Purified pneumococcal polysaccharides of types 19A and 19F were manufactured by Pfizer and purchased through the American Type Culture Collection (ATCC 301-X® and ATCC 84-X®). Dot-blot analyses were performed essentially as described previously by Van Calsteren *et al.* (15). Ten microliters of purified native CPS (each at 1 mg/ml in water) of *S. suis* serotype 2 (used as control and purified as described (13)), *S. suis* serotype 8 and pneumococcal types 19A and 19F were blotted on a PVDF Western blot membrane (Bio-Rad, Hercules, CA). The membrane was blocked for 1 h with a solution of Tris-buffered saline (TBS) containing 2% skim milk, followed by 2 h of incubation with monospecific polyclonal rabbit serum (18). The membrane was washed three times with TBS, and anti-rabbit HRP-conjugated antibody (Jackson Immunoresearch, West Grove, PA) was added for 1 h. The membrane was washed three times with TBS and revealed with 4-chloro-1-naphthol solution (Sigma-Aldrich, Oakville, ON, Canada). Densitometric analysis of the dot-blot scan was performed using ImageJ version 1.49 (19). Raw integration values were normalized using the homologous signal value (serotype 8).

3. Results and Discussion

3.1. Capsular polysaccharide production and purification for serotypes 7 and 8

Using field strains of *S. suis* serotype 7 (#1750775) and of serotype 8 (#1719887), 46 mg and 40 mg, respectively, of CPS were obtained after extraction and purification by gel filtration from 6 L of fermentation broth in one experiment each. For serotype 7, the CPS was partially hydrolyzed, but a significant amount of the polymeric fraction was collected, as well as its repeating unit, its dimer and trimer. In contrast, the serotype 8 CPS was not found to be degraded after purification. Nucleic acid analysis confirmed the absence of significant contamination with 0.1% and 0.2% w/w DNA/RNA for serotypes 7 and 8, respectively. Similarly, protein levels were found to be under the detection limit (< 0.7% w/w) in both purified CPS.

3.2. Structure determination and NMR for serotype 7

The purified serotype 7 CPS was analyzed by 2D NMR using COSY, TOCSY, ROESY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, ^{31}P, and ^{1}H-^{31}P HSQC experiments. Monosaccharides were identified based on NMR signal positions and coupling constants in agreement with the presented structure (Fig. 1 and Table 1). The CPS had a regular structure and contained 4 sugars: α-D-Gal, α-L-Rha, β-D-GlcA, α-D-FucNAc4N, and one phosphate residue per repeating unit. All sugars were in pyranose form. Rha H-1 had $J_{P\text{-}H}$ 7 Hz, correlating in ^{1}H-^{31}P HMQC with ^{31}P signal at -2.7 ppm, which also correlated with H-2 of α-Gal, indicating linkage B1-*P*-A2. C-6 of glucuronic acid was identified by HMBC correlation H-5:C-6, H-4:C-6. The FucNAc4N residue was acetylated at N-2, identified by HMBC correlation H-2 of C to CO of the acetate; no acetate was present at N-4 of this sugar. The sequence of C-B-A monosaccharides was determined by 2D NMR based on the NOE and HMBC interresidual correlations A1:D4; D1:C3; C1:B3. Linkage B1-*P*-A2 was determined from ^{1}H-^{31}P correlation as mentioned above. Mild hydrolysis of the polymer (2% AcOH, 100°, 1 h) partially cleaved the linkage between Rha and phosphate; sugar phosphates are highly susceptible to hydrolysis (20).

Absolute configurations of D-Gal, L-Rha, D-GlcA were determined by GLC-MS of 2-octyl glycosides acetates with optically pure 2-octanol. Absolute D-configuration of FucNAc4N was proven by comparison of experimental and calculated chemical shifts for the same sugar sequence with D- or L-FucN4N using averaged experimental database (21).

Taken together, these results yielded the following tetrasaccharide repeating unit for the serotype 7 CPS:

$$[3)\text{-}\alpha\text{-L-Rha}p\text{-}1\rightarrow P\rightarrow 2\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-GlcA}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Fuc}p\text{NAc4N-}(1\rightarrow]_n$$

B A D C

This report is the first description of the sugar sequence of *S. suis* serotype 7. This CPS possesses negatively charged glucuronic acid and phosphate and a positively charged aminogroup of 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (FucNAc4N). Still, like all

described CPSs of *S. suis* (serotype 8 included), they are all found to be acidic (negatively charged) due to the presence of either phosphates, sialic acids (Neu5Ac) or uronic acids (Supplementary Fig. S1). Another unusual feature of this polysaccharide is the presence of the FucNAc4N sugar, a rare diamino sugar that was reported in the lipopolysaccharide of *Bordetella hinzii* (22) and in a secondary cell wall polymer of *Bacillus cereus*, where it was shown that its expression was tightly regulated by culture conditions and growth states (23).

3.3. Structure determination and NMR for serotype 8

The purified serotype 8 CPS was analyzed by 2D NMR using COSY, TOCSY, ROESY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, ^{31}P, and ^{1}H-^{31}P HSQC experiments. Monosaccharides were identified as alditol acetates and absolute configurations were determined by GLC-MS of 2-octyl glycosides acetates with optically pure 2-octanol (Fig. 2 and Table 2). The CPS had a regular structure and contained three sugars: α-D-Glc, α-L-Rha, β-D-ManNAc, and one phosphate residue per repeating unit. All sugars were in pyranose form. All sugars had NMR signal positions and coupling constants in agreement with the presented structure. Rha H-1 had $J_{P\text{-}H}$ 7 Hz, correlating in ^{1}H-^{31}P HMQC with ^{31}P signal at -1.6 ppm, which also correlated with H-4 of ManNAc, which indicated the linkage A1-*P*-C4. The sequence of C-B-A monosaccharides was determined by 2D NMR based on the NOE and HMBC interresidual correlations B1:A2; C1:B4.

This CPS was found to be unstable in water solution and signals of the hydrolysis products of the Rha-P linkage were growing significantly during spectra recording. Fresh polysaccharide solution showed only NMR signals of non-hydrolyzed polymer. ^{31}P signal in terminal ManNAc4*P* ('C') was at 1.2 ppm.

P-4-β-ManNAc-4-α-Glc-2-α-Rha-1-*P*-**[4-β-ManNAc-4-α-Glc-2-α-Rha-*P*-]**$_n$-4-β-ManNAc-4-α-Glc-2-α,β-Rha
C’ C B A B’ A’

Taken together, these results yielded the following trisaccharide repeating unit for the serotype 8 CPS:

$$[2)\text{-}\alpha\text{-}L\text{-}Rha\textit{p}\text{-}1\rightarrow\textit{P}\rightarrow 4\text{-}\beta\text{-}D\text{-}Man\textit{p}NAc\text{-}(1\rightarrow 4)\text{-}\alpha\text{-}D\text{-}Glc\textit{p}\text{-}(1\rightarrow]_n$$

A C B

This report is also the first description of the sugar sequence of *S. suis* serotype 8. Based on the CPS structures reported for 9 out of the 35 serotypes of *S. suis*, it might appear that overall the CPSs of *S. suis* exhibit a high structural variety similar to the large diversity observed in *S. pneumoniae* (24), as some of those CPSs contain phosphates, sialic acids, uronic acids and/or other unusual sugars (such as FucNAc4N, di-*N*-acetyl-bacillosamine, glucitol, and 2-acetamido-2,6-dideoxy-*xylo*-hexopyranos-4-ulose) (Supplementary Fig. S1). On the other hand, the CPSs of Group B *Streptococcus* are much more limited in their structural diversity. All of its 10 serotypes possess an α2,3-linked sialic acid and are formed by different arrangements of Glc, GlcNAc, Gal, and Rha (Supplementary Fig. S2). Interestingly, the structures of the α2,6-sialylated CPSs of *S. suis* (namely serotypes 2, 1/2, 14, and 1) are closely related to those of Group B *Streptococcus* (Supplementary Figs. S1 and S2), while those of the other serotypes are well diverse (namely serotypes 3, 7, 8, 9, and 18).

3.4. Relationship of S. suis serotype 8 CPS to pneumococcal group 19

In 1984, Lee *et al.* published their study into the cross-reactivity of nongroupable streptococci with pneumococcal group 19 (25). Most notably, they observed that the crude CPS preparation of *S. suis* strain 14636 (the reference strain for serotype 8 (26)) reacted strongly with pneumococcal type 19F, and that it also possessed the same chemical composition. Finally, without conducting a full structural assignment of the serotype 8 CPS, they concluded by comparing ^{13}C-NMR spectra that *S. suis* serotype 8 CPS is identical to pneumococcal type 19F. Then, in 1995, Gottschalk *et al.* confirmed the previous results by employing two monoclonal antibodies (mAbs) raised against pneumococcal group 19 (19A and 19F) and showing that these mAbs reacted specifically with serotype 8 strains of *S. suis* (27).

Since our results herein also confirm that *S. suis* serotype 8 and pneumococcal type 19F both possess the same repeating unit structure for their CPSs, we performed dot-blot

experiments to investigate the reactivity of anti-*S. suis* serotype 8 rabbit antisera with the purified 19A and 19F pneumococcal polysaccharides (Fig. 3). While serotype 8 antisera reacted with both 19A and 19F polysaccharides (Fig. 3A), densitometric analysis shows that the reaction is stronger with 19F than with 19A (Fig. 3B), as can be expected based on their known structures (Fig. 3C).

3.5. Assignment of functions of the cps locus genes involved in serotypes 7 and 8 CPS biosynthesis

S. suis CPS is considered to be synthesized via the Wzx/Wzy-dependent pathway (28). In this pathway, the initial glycosyltransferase transfers the first sugar to a membrane-associated lipid carrier (assumed to be undecaprenyl phosphate [Und-P]), and then specific glycosyltransferases sequentially link additional sugars to form a repeating unit on the inner face of the cytoplasmic membrane. Wzx flippase subsequently transports the repeating unit to the outer surface of the cytoplasmic membrane, and Wzy polymerase polymerizes the repeating units to generate mature CPS. Polymerized CPS is then translocated to the peptidoglycan by the membrane protein complex (29). Genes responsible for this pathway are clustered in the *cps* locus on the *S. suis* chromosome, and the first four genes (*cpsABCD*) in the loci, which are considered to be involved in the regulation and processing of CPS, are conserved in all serotypes (28). Based on the CPS structures determined in this study, *cps* genes involved in the serotype 7 and 8 CPS biosynthesis were tentatively assigned (Tables 3 and 4 and Fig. 4). For the assignment, BLAST analysis (30) and conserved domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (31) were carried out by using sequence data on the *cps* gene clusters of *S. suis* serotypes 7 and 8 reference strains (GenBank accession nos. BR001004 and BR001005, respectively) (28).

Since the repeating unit of the serotype 7 CPS is composed of four sugars (FucNAc4N, GlcA, Gal, and Rha) and one phosphate residue, four glycosyltransferases and one phosphotransferase are assumed to be involved in its assembly. As expected, the conserved domain search predicted the presence of four glycosyltransferase genes, *cps7F*, *cps7H*, *cps7I*, and *cps7K*, in the *cps* locus of serotype 7. Among the products encoded by the genes, Cps7F showed similarity with glycosyltransferases WbgY of *Shigella sonnei* 53G (amino acid

identity, 44%; coverage, 86%) and WcfS of *Bacteroides fragilis* NCTC 9343 (amino acid identity, 49%; coverage, 87%). Because WbgY and WcfS are proposed to be responsible for the transfer of the first sugar FucNAc4N to the lipid carrier in the O-antigen (32) and CPS (33) repeating unit synthesis pathways, respectively, we regarded FucNAc4N as the first sugar of the repeating unit of serotype 7 CPS and Cps7F as the initial glycosyltransferase for the transfer of the first sugar to Und-P (Fig. 4A and Table 3). In *S. sonnei* 53G, nucleoside-diphosphate sugar epimerase WbgZ and aminotransferase WbgX were predicted to be involved in the synthesis of UDP-FucNAc4N (32). In *S. suis* serotype 7, Cps7E and Cps7G had approximately 40% amino acid identities to WbgZ and WbgX of *S. sonnei* 53G, respectively. Furthermore, Cps7G was also similar to the C4″- aminotransferase (amino acid identity, 62%; coverage, 98%) involved in UDP-FucNAc4N synthesis in *Bacillus cereus* ATCC 14579 (34). These results suggest that Cps7E and Cps7G are responsible for the synthesis of UDP-FucNAc4N in *S. suis* serotype 7 (Fig. 4A and Table 3).

Given the above discussion, we regarded GlcA, Gal, phosphate, and Rha as the second, third, fourth and fifth components, respectively, in the serotype 7 CPS repeating unit (Fig. 4A). Among the other three glycosyltransferases, Cps7K showed some similarity to rhamnosyl transferases of *S. pneumoniae* strains (amino acid identity, 41-45%; coverage, 98%), suggesting that Cps7K transfers Rha to the phosphate residue (Fig. 4A and Table 3). Our homology search did not infer the substrates of glycosyltransferases Cps7H and Cps7I nor the presence of phosphotransferases in the *cps* locus; however, given that transferases work in the order of genes in the *cps* locus, Cps7H and Cps7I may be involved in the addition of GlcA to FucNAc4N and Gal to GlcA, respectively, and Cps7J encoded between *cps7I* and *cps7K* may transfer the phosphate residue to Gal (Fig. 4A and Table 3), although further study is necessary to verify this hypothesis. By the blastp and conserved domain searches, we assigned Cps7L and Cps7M to the polymerase Wzy and flippase Wzx, respectively (Table 3). Cps7N was assigned to UDP-glucose 6-dehydrogenase Ugd, which is known to catalyze UDP-GlcA synthesis (35).

As the CPS repeating unit structure of *S. suis* serotype 8 was identical to that of *S. pneumoniae* serotype 19F, we compared the amino acid sequences of *cps* gene products

between *S. suis* 14636 (serotype 8) and *S. pneumoniae* 485/61(serotype 19F) by blastp and tblastx (Table 4 and Fig. S3). The repeating unit is composed of three sugars (Glc, ManNAc, and Rha) and one phosphate residue. Three glycosyltransferases (WchA, WchO, and WchQ) and one phosphotransferase WchP are encoded in the *cps* locus of *S. pneumoniae* serotype 19F. WchA is the initial glycosyltransferase and is predicted to transfer Glc to Und-P; therefore, Glc is the first sugar in the CPS repeating unit, and ManNAc, phosphate and Rha are considered to be the second, third, and fourth components, respectively (29). Because WchQ is annotated as a putative rhamnosyl transferase, WchO may add ManNAc to Glc as the second glycosyltransferase, and then WchP and WchQ may catalyze the transfer of a phosphate residue to ManNAc and Rha to the phosphate residue, respectively. Among the *cps* gene products of *S. suis* serotype 8, Cps8E, Cps8H, Cps8I, and Cps8J showed more than 50% amino acid sequence identities with WchA, WchO, WchP, and WchQ of *S. pneumoniae* serotype 19F, respectively (Table 4); therefore, we tentatively assigned that Cps8E is the initial glycosyltransferase catalyzing the transfer of Glc to Und-P, and then Cps8H, Cps8I, and Cps8J sequentially add ManNAc, phosphate and Rha in the repeating unit, respectively (Table 4 and Fig. 4B). In addition to these glycosyltransferases, Cps8K, Cps8L, and Cps8M also had similarity with *cps* gene products of *S. pneumoniae* serotype 19F (Table 4). Cps8K and Cps8M were predicted to be the repeating unit polymerase Wzy and flippase Wzx, respectively. Cps8L was inferred to be the UDP-N-acetylglucosamine-2-epimerase, an enzyme which catalyzes the conversion of UDP-GlcNAc to UDP-ManNAc (36). Cps8F, Cps8G, Cps8N, Cps8O, and Cps8P homologues were not found in the *cps* locus of *S. pneumoniae* serotype 19F (Fig. S3), and their role in CPS biosynthesis is unknown.

This study provides the basis for further studies on important structural elements recognized by anti-CPS specific antibodies, and thus improve our understanding of the serological reactions leading to *S. suis* serotype classification. The CPS is a proven major virulent determinant for *S. suis* serotypes 2 and 14, which protects the bacteria against phagocytosis and impairs host immune activation (4, 37-39). Since then, varied CPS structures have been described for other serotypes and studies are being performed to evaluate their role in virulence. Knowledge of CPS structure and composition will contribute to better dissect the role of this bacterial component in the pathogenesis of *S. suis* serotypes 7 and 8 by means of

mutagenesis studies, immunogenicity studies, as well as immunological studies of CPS-mediated modulation of antigen-presenting cells and B lymphocytes, in comparison to previously described *S. suis* CPSs (40-42).

Acknowledements

The authors thank Ms. Claudia Duquette and Ms. Léa Martelet for excellent technical assistance during capsular polysaccharide production and purification. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a grant to MS (#342150) and partial support by the Fonds de recherche du Québec—Nature et technologies (FRQ-NT) (grant # 2018-PR-206110 to MS and MG). GGD is a recipient of a FRQNT Doctoral Scholarship and an Alexander Graham Bell Graduate Scholarship—Doctoral Program from NSERC.

References

1. Gottschalk M. Streptococcosis. In: Zimmerman JJ, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of swine. 10th ed. Ames, USA: Wiley-Blackwell Publishing; 2012. p. 841-55.
2. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect. 2014;3(6):e45.
3. Fittipaldi N, Segura M, Grenier D, Gottschalk M. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent *Streptococcus suis*. Future Microbiol. 2012;7(2):259-79.
4. Roy D, Auger JP, Segura M, Fittipaldi N, Takamatsu D, Okura M, et al. Role of the capsular polysaccharide as a virulence factor for *Streptococcus suis* serotype 14. Can J Vet Res. 2015;79(2):141-6.
5. Gottschalk M, Lacouture S. Canada: Distribution of *Streptococcus suis* (from 2012 to 2014) and *Actinobacillus pleuropneumoniae* (from 2011 to 2014) serotypes isolated from diseased pigs. Can Vet J. 2015;56(10):1093-4.
6. Wongsawan K, Gottschalk M, Tharavichitkul P. Serotype- and virulence-associated gene profile of *Streptococcus suis* isolates from pig carcasses in Chiang Mai Province, Northern Thailand. J Vet Med Sci. 2015;77(2):233-6.
7. Nutravong T, Angkititrakul S, Jiwakanon N, Wongchanthong W, Dejsirilerts S, Nawa Y. Identification of major *Streptococcus suis* serotypes 2, 7, 8 and 9 isolated from pigs

and humans in upper northeastern Thailand. Southeast Asian J Trop Med Public Health. 2014;45(5):1173-81.
8. Thongkamkoon P, Kiatyingangsulee T, Gottschalk M. Serotypes of *Streptococcus suis* isolated from healthy pigs in Phayao Province, Thailand. BMC Res Notes. 2017;10(1):53.
9. Oh SI, Jeon AB, Jung BY, Byun JW, Gottschalk M, Kim A, et al. Capsular serotypes, virulence-associated genes and antimicrobial susceptibility of *Streptococcus suis* isolates from pigs in Korea. J Vet Med Sci. 2017;79(4):780-7.
10. Gottschalk M, Xu J, Lecours M-P, Grenier D, Fittipaldi N, Segura M. *Streptococcus suis* Infections in Humans: What is the prognosis for Western countries? (Part II). Clin Microbiol Newsl. 2010;32(13):97-102.
11. Unterweger C, Baums CG, Hocher M, Fischer L, Weiss A, Hennig-Pauka I. [Clinical situation, diagnosis and prevention of a *Streptococcus suis* serotype 7 problem on a farm]. Berl Munch Tierarztl Wochenschr. 2014;127(5-6):194-201.
12. Unterweger C, Ruczizka U, Spergser J, Baums CG, Hennig-Pauka I. Effect of early-life treatment of piglets with long-acting ceftiofur on colonization of *Streptococcus suis* serotype 7 and elicitation of specific humoral immunity in a farm dealing with streptococcal diseases. Pathogens. 2018;7(2):34.
13. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. Biochem Cell Biol. 2010;88(3):513-25.
14. Van Calsteren MR, Gagnon F, Calzas C, Goyette-Desjardins G, Okura M, Takamatsu D, et al. Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide. Biochem Cell Biol. 2013;91(2):49-58.
15. Van Calsteren MR, Goyette-Desjardins G, Gagnon F, Okura M, Takamatsu D, Roy R, et al. Explaining the serological characteristics of *Streptococcus suis* serotypes 1 and 1/2 from their capsular polysaccharide structure and biosynthesis. J Biol Chem. 2016;291(16):8387-98.
16. Vinogradov E, Goyette-Desjardins G, Okura M, Takamatsu D, Gottschalk M, Segura M. Structure determination of *Streptococcus suis* serotype 9 capsular polysaccharide and assignment of functions of the cps locus genes involved in its biosynthesis. Carbohydr Res. 2016;433:25-30.
17. Goyette-Desjardins G, Vinogradov E, Okura M, Takamatsu D, Gottschalk M, Segura M. *Streptococcus suis* serotype 3 and serotype 18 capsular polysaccharides contain di-N-acetyl-bacillosamine. Carbohydr Res. 2018;466:18-29.
18. Higgins R, Gottschalk M. An update on *Streptococcus suis* identification. J Vet Diagn Invest. 1990;2(3):249-52.
19. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature methods. 2012;9(7):671-5.
20. Mikkola S. Hydrolysis and isomerization of sugar phosphates and carbohydrate phosphodiesters. Curr Org Chem. 2013;17(14):1525-44.
21. Lipkind GM, Shashkov AS, Knirel YA, Vinogradov EV, Kochetkov NK. A computer-assisted structural analysis of regular polysaccharides on the basis of 13C-n.m.r. data. Carbohydr Res. 1988;175(1):59-75.

22. Aussel L, Chaby R, Le Blay K, Kelly J, Thibault P, Perry MB, et al. Chemical and serological characterization of the *Bordetella hinzii* lipopolysaccharides. FEBS Lett. 2000;485(1):40-6.
23. Candela T, Maes E, Garenaux E, Rombouts Y, Krzewinski F, Gohar M, et al. Environmental and biofilm-dependent changes in a *Bacillus cereus* secondary cell wall polysaccharide. J Biol Chem. 2011;286(36):31250-62.
24. Geno KA, Gilbert GL, Song JY, Skovsted IC, Klugman KP, Jones C, et al. Pneumococcal capsules and their types: past, present, and future. Clin Microbiol Rev. 2015;28(3):871-99.
25. Lee CJ, Koizumi K, Henrichsen J, Perch B, Lin CS, Egan W. Capsular polysaccharides of nongroupable streptococci that cross-react with Pneumococcal Group 19. J Immunol. 1984;133(5):2706-11.
26. Perch B, Pedersen KB, Henrichsen J. Serology of capsulated streptococci pathogenic for pigs: six new serotypes of *Streptococcus suis*. J Clin Microbiol. 1983;17(6):993-6.
27. Gottschalk M, Kolberg J, Charland N, Jacques M. *Streptococcus pneumoniae* types 19A and 19F and *Streptococcus suis* capsular type 8 share common capsular epitopes. J Clin Microbiol. 1995;33(9):2492-5.
28. Okura M, Takamatsu D, Maruyama F, Nozawa T, Nakagawa I, Osaki M, et al. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: potential mechanisms for generation of capsular variation. Appl Environ Microbiol. 2013;79(8):2796-806.
29. Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, Collins M, et al. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet. 2006;2(3):e31.
30. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. Nucleic Acids Res. 2008;36(Web Server issue):W5-9.
31. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, et al. CDD: NCBI's conserved domain database. Nucleic Acids Res. 2015;43(Database issue):D222-6.
32. Xu DQ, Cisar JO, Ambulos N, Jr., Burr DH, Kopecko DJ. Molecular cloning and characterization of genes for *Shigella sonnei* form I O polysaccharide: proposed biosynthetic pathway and stable expression in a live *salmonella* vaccine vector. Infect Immun. 2002;70(8):4414-23.
33. Coyne MJ, Tzianabos AO, Mallory BC, Carey VJ, Kasper DL, Comstock LE. Polysaccharide biosynthesis locus required for virulence of *Bacteroides fragilis*. Infect Immun. 2001;69(7):4342-50.
34. Hwang S, Li Z, Bar-Peled Y, Aronov A, Ericson J, Bar-Peled M. The biosynthesis of UDP-d-FucNAc-4N-(2)-oxoglutarate (UDP-Yelosamine) in *Bacillus cereus* ATCC 14579: Pat and Pyl, an aminotransferase and an ATP-dependent Grasp protein that ligates 2-oxoglutarate to UDP-4-amino-sugars. J Biol Chem. 2014;289(51):35620-32.
35. Druzhinina TN, Kusov YY, Shibaev VN, Kochetkov NK, Biely P, Kucar S, et al. Uridine diphosphate 2-deoxyglucose. Chemical synthesis, enzymic oxidation and epimerization. Biochim Biophys Acta. 1975;381(2):301-7.
36. Meier-Dieter U, Starman R, Barr K, Mayer H, Rick PD. Biosynthesis of enterobacterial common antigen in *Escherichia coli*. Biochemical characterization of Tn10 insertion

mutants defective in enterobacterial common antigen synthesis. J Biol Chem. 1990;265(23):13490-7.
37. Charland N, Harel J, Kobisch M, Lacasse S, Gottschalk M. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology. 1998;144 (Pt 2):325-32.
38. Smith HE, Damman M, van der Velde J, Wagenaar F, Wisselink HJ, Stockhofe-Zurwieden N, et al. Identification and characterization of the cps locus of *Streptococcus suis* Serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect Immun. 1999;67(4):1750-6.
39. Auger JP, Dolbec D, Roy D, Segura M, Gottschalk M. Role of the *Streptococcus suis* serotype 2 capsular polysaccharide in the interactions with dendritic cells is strain-dependent but remains critical for virulence. PLoS One. 2018;13(7):e0200453.
40. Calzas C, Goyette-Desjardins G, Lemire P, Gagnon F, Lachance C, Van Calsteren MR, et al. Group B *Streptococcus* and *Streptococcus suis* capsular polysaccharides induce chemokine production by dendritic cells via Toll-like receptor 2- and MyD88-dependent and -independent pathways. Infect Immun. 2013;81(9):3106-18.
41. Calzas C, Lemire P, Auray G, Gerdts V, Gottschalk M, Segura M. Antibody response specific to the capsular polysaccharide is impaired in *Streptococcus suis* serotype 2-infected animals. Infect Immun. 2015;83(1):441-53.
42. Calzas C, Taillardet M, Fourati I, Roy D, Gottschalk M, Soudeyns H, et al. Evaluation of the immunomodulatory properties of *Streptococcus suis* and Group B *Streptococcus* capsular polysaccharides on the humoral response. Pathogens. 2017;6(2):16.
43. Katzenellenbogen E, Jennings HJ. Structural determination of the capsular polysaccharide of *Streptococcus pneumoniae* type 19A (57). Carbohydr Res. 1983;124(2):235-45.
44. Jennings HJ, Rosell K-G, Carlo DJ. Structural determination of the capsular polysaccharide of *Streptococcus pneumoniae* type 19 (19F). Can J Chem. 1980;58(11):1069-74.
45. Varki A, Cummings RD, Aebi M, Packer NH, Seeberger PH, Esko JD, et al. Symbol Nomenclature for Graphical Representations of Glycans. Glycobiology. 2015;25(12):1323-4.
46. Jennings HJ, Katzenellenbogen E, Lugowski C, Kasper DL. Structure of native polysaccharide antigens of type Ia and type Ib Group B *Streptococcus*. Biochemistry. 1983;22(5):1258-64.
47. Jennings HJ, Rosell KG, Katzenellenbogen E, Kasper DL. Structural determination of the capsular polysaccharide antigen of type II Group B *Streptococcus*. J Biol Chem. 1983;258(3):1793-8.
48. Jennings HJ, Lugowski C, Kasper DL. Conformational aspects critical to the immunospecificity of the type III Group B streptococcal polysaccharide. Biochemistry. 1981;20(16):4511-8.
49. Wessels MR, Pozsgay V, Kasper DL, Jennings HJ. Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III Group B *Streptococcus*. A revised structure for the type III Group B streptococcal polysaccharide antigen. J Biol Chem. 1987;262(17):8262-7.
50. Di Fabio JL, Michon F, Brisson J-R, Jennings HJ, Wessels MR, Benedí V-J, et al. Structure of the capsular polysaccharide antigen of type IV Group B *Streptococcus*. Can J Chem. 1989;67(5):877-82.

51. Wessels MR, DiFabio JL, Benedi VJ, Kasper DL, Michon F, Brisson JR, et al. Structural determination and immunochemical characterization of the type V Group B *Streptococcus* capsular polysaccharide. J Biol Chem. 1991;266(11):6714-9.
52. von Hunolstein C, D'Ascenzi S, Wagner B, Jelinkova J, Alfarone G, Recchia S, et al. Immunochemistry of capsular type polysaccharide and virulence properties of type VI *Streptococcus agalactiae* (Group B streptococci). Infect Immun. 1993;61(4):1272-80.
53. Kogan G, Brisson JR, Kasper DL, von Hunolstein C, Orefici G, Jennings HJ. Structural elucidation of the novel type VII Group B *Streptococcus* capsular polysaccharide by high resolution NMR spectroscopy. Carbohydr Res. 1995;277(1):1-9.
54. Kogan G, Uhrin D, Brisson JR, Paoletti LC, Blodgett AE, Kasper DL, et al. Structural and immunochemical characterization of the type VIII Group B *Streptococcus* capsular polysaccharide. J Biol Chem. 1996;271(15):8786-90.
55. Berti F, Campisi E, Toniolo C, Morelli L, Crotti S, Rosini R, et al. Structure of the type IX Group B *Streptococcus* capsular polysaccharide and its evolutionary relationship with types V and VII. J Biol Chem. 2014;289(34):23437-48.
56. Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J. ACT: the Artemis Comparison Tool. Bioinformatics. 2005;21(16):3422-3.

Tables

Table 1. NMR data for the native polysaccharide of *S. suis* serotype 7 capsular polysaccharide.[1]

Unit		1	2	3	4	5	6
→2)-α-D-Gal-(1→	H	5.69	4.26	3.90	4.03	3.95	3.69; 3.69
A	C	97.9	74.0	69.3	70.0	71.4	61.5
→3)-α-L-Rha-(1→	H	5.45	4.15	3.87	3.55	3.95	1.34
B	C	97.2	68.5	76.7	71.3	71.1	18.1
→3)-α-D-FucNAc4N-(1→	H	5.10	4.23	4.35	3.90	4.64	1.27
C	C	95.8	49.0	75.8	56.1	63.9	16.5
→4)-β-D-GlcA-(1→	H	4.62	3.37	3.80	3.80	3.86	
D	C	105.4	73.4	77.8	77.8	77.1	176.1

[1] Bruker Avance III 600 MHz, at 25°C. NAc at 2.04/23.2; 175.9 ppm. Acetone was used as an internal reference (2.225 ppm for ^{1}H and 31.45 ppm for ^{13}C).

Table 2. NMR data for the native polysaccharide of *S. suis* serotype 8 capsular polysaccharide.[1]

Unit		1	2	3	4	5	6
→2)-α-L-Rha-(1→*P*	H	5.50	4.03	3.93	3.52	3.89	1.31
A	C	95.0	78.0	70.2	72.8	71.3	17.9
→2)-α-L-Rha	H	5.23	3.93	3.89	3.50	3.88	1.29
A’	C	92.7	78.5	70.4	73.2	69.8	17.9
→2)-β-L-Rha	H	4.94	4.00	3.66	3.37	3.43	1.30
A’	C	94.9	82.0	73.1	73.0	73.4	17.9
→4)-α-D-Glc-(1→	H	5.02	3.58	3.90	3.70	4.09	3.76; 3.76
B	C	98.9	72.2	72.4	79.8	71.5	60.9
→4)-α-D-Glc-(1→2)-α-L-Rha	H	5.00	3.57	3.90	3.69	4.09	3.76; 3.76
B’-A’	C	98.9	72.3	72.4	79.8	71.5	60.9
→4)-α-D-Glc-(1→2)-β-L-Rha	H	5.09	3.62	3.93	3.71	4.09	3.76; 3.76
B’-A’	C	102.1	73.0	72.6	79.8	71.5	60.9
P→4)-β-D-ManNAc-(1→	H	4.92	4.58	4.00	4.08	3.56	3.86; 3.94
C	C	100.5	54.3	72.5	73.3	76.8	61.6
P→4)-β-D-ManNAc-(1→	H	4.92	4.58	4.00	4.05	3.56	3.86; 3.94
C’	C	100.5	54.3	72.5	72.6	76.8	61.6

[1] Bruker Avance III 600 MHz, at 25°C. NAc at 2.09/23.2; 176.7 ppm. Acetone was used as an internal reference (2.225 ppm for ^{1}H and 31.45 ppm for ^{13}C).

Table 3. Putative function of *cps* gene products of *S. suis* serotype 7 reference strain involved in the synthesis of its capsular polysaccharide.

Locus tag	Conserved domain	Product (putative)	Putative Function/Pathway	Description
cps7A	pfam03816: LytR_cpsA_psr; Cell envelope-related transcriptional attenuator domain pfam02916: DNA_PPF; DNA polymerase processivity factor	Integral membrane regulatory protein Wzg	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps7B	cl26080: GNVR super family; G-rich domain on putative tyrosine kinase	Chain length determinant protein/polysaccharide export protein Wzd	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps7C	TIGR01007: eps_fam; Capsular exopolysaccharide family	Tyrosine-protein kinase Wze	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps7D	cl23724: PHP super family; Polymerase and histidinol phosphatase domain	Protein-tyrosine phosphatase Wzh	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps7E	COG1086: FlaA1; NDP-sugar	Nucleoside-diphosphate	UDP-FucNAc4N	Similar to FucNAc4N synthesis

	epimerase	sugar epimerase	synthesis	protein WbgZ of *Shigella sonnei* 53G (amino acid identity, 39%; coverage, 90%)
cps7F	pfam02397: Bac_transf; Bacterial sugar transferase	Glycosyltransferase	Addition of FucNAc4N to the lipid carrier (Initial glycosyltransferase)	Similar to FucNAc4N transferases WcfS of *Bacteroides fragilis* NCTC 9343 (amino acid identity, 49%; coverage, 87%), and WbgY of *S. sonnei* 53G (amino acid identity, 44%; coverage, 86%)
cps7G	cd00616: AHBA_syn; 3-amino-5-hydroxybenzoic acid synthase family	Aminotransferase	UDP-FucNAc4N synthesis	Similar to FucNAc4N synthesis proteins WcfR of *B. fragilis* NCTC 9343 (amino acid identity, 45%; coverage, 96%) and WbgX of *S. sonnei* 53G (amino acid identity, 37%; coverage, 96%) Similar to FucNAc4N synthesis protein Pat of *Bacillus cereus* ATCC 14579 (amino acid identity, 62%; coverage, 98%)

cps7H	pfam00535: Glycos_transf_2; Glycosyl transferase family 2 COG0463: WcaA; Glycosyltransferase involved in cell wall biosynthesis	Glycosyltransferase	Addition of GlcA to FucNAc4N?	
cps7I	COG0438: RfaB; Glycosyltransferase involved in cell wall biosynthesis	Glycosyltransferase	Addition of Gal to GlcA?	
cps7J	TIGR00368: TIGR00368; Mg chelatase-related protein (the N-terminal)	Hypothetical protein	Addition of phosphate to Gal?	Similar to ATP-dependent zinc metalloprotease FtsH of several species (amino acid identity, 22-27%; coverage, 41-52%)
cps7K	cl10013:GT2; Glycosyltransferase, GT2 family	Glycosyltransferase	Addition of Rha to phosphate	Similar to putative rhamnosyl transferase WchQ of *Streptococcus pneumoniae* serotype 19A, 19F, 32A and 32F strains (amino acid identity, 41-45%; coverage, 98%)
cps7L	TIGR04370: glyco_rpt_poly; Oligosaccharide repeat unit polymerase	Oligosaccharide repeat unit polymerase Wzy	Polymerization of repeat units	

cps7M	cd13128: MATE_Wzx_like; Wzx, a subfamily of the multidrug and toxic compound extrusion (MATE)-like proteins pfam14667: Polysacc_synt_C; Polysaccharide biosynthesis C-terminal domain	Flippase Wzx	Transportation of repeat units	
cps7N	cl27617: UDPG_MGDP_dh super family; UDP-glucose/GDP-mannose dehydrogenase family, central domain	UDP-glucose dehydrogenase	UDP-GlcA synthesis	Similar to UDP-glucose 6-dehydrogenase Ugd of *S. pneumoniae* (amino acid identity, 55-74%; coverage, 41-75%)
cps7O	cl18945: AAT_I super family; Aspartate aminotransferase superfamily (fold type I) of pyridoxal phosphate-dependent enzyme	Serine hydroxymethyltransferase (probable pseudogene)	Unknown	
cps7P		Serine hydroxymethyltransferase (probable pseudogene)	Unknown	

cps7Q	pfam13424: TPR_12; Tetratricopeptide repeat	Hypothetical protein	Unknown
cps7R	pfam13191: AAA_16; AAA ATPase domain	Hypothetical protein	Unknown

Table 4. Putative function of *cps* gene products of *S. suis* serotype 8 reference strain involved in the synthesis of its capsular polysaccharide.

Locus tag	Conserved domain	Product (putative)	Putative Function/Pathway	Description
cps8A	pfam03816: LytR_cpsA_psr; Cell envelope-related transcriptional attenuator domain pfam02916: DNA_PPF; DNA polymerase processivity factor	Integral membrane regulatory protein Wzg	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps8B	cl26080: GNVR super family; G-rich domain on putative tyrosine kinase	Chain length determinant protein/polysaccharide export protein Wzd	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps8C	TIGR01007: eps_fam; Capsular exopolysaccharide family	Tyrosine-protein kinase Wze	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*
cps8D	cl23724: PHP super family; Polymerase and histidinol phosphatase domain	Protein-tyrosine phosphatase Wzh	CPS synthesis (regulation?)	Conserved in all types of *cps* gene cluster in *S. suis*

cps8E	TIGR03025: EPS_sugtrans; Exopolysaccharide biosynthesis polyprenyl glycosylphosphotransferase	Glycosyltransferase	Addition of Glc to the lipid carrier (Initial glycosyltransferase)	Similar to undecaprenylphosphate glucosephosphotransferase WchA (initial glycosyltransferase) of *Streptococcus pneumoniae* serotype 19F (amino acid identity, 56%; coverage, 97%)
cps8F	cl27287: GLF super family; UDP-galactopyranose mutase	UDP-galactopyranose mutase (probable pseudogene)		
cps8G	pfam03275: GLF; UDP-galactopyranose mutase	UDP-galactopyranose mutase (probable pseudogene)		
cps8H	cd06533: Glyco_transf_WecG_TagA; The glycosyltransferase WecG/TagA superfamily contains *Escherichia coli* WecG, *Bacillus subtilis* TagA and related proteins.	Glycosyltransferase	Addition of ManNAc to Glc	Similar to glycosyltransferase WchO of *S. pneumoniae* serotype 19F (amino acid identity, 66%; coverage, 97%)
cps8I	pfam04991: LicD; LicD family of proteins involved in	LicD-family phosphotransferase	Addition of phosphate to	Similar to LicD-family phosphotransferase WchP of *S.*

	phosphorylcholine metabolism		ManNAc	*pneumoniae* serotype 19F (amino acid identity, 66%; coverage, 99%)
cps8J	COG1216: GT2; Glycosyltransferase, GT2 family	Glycosyltransferase	Addition of Rha to phosphate	Similar to putative rhamnosyl transferase WchQ of *S. pneumoniae* serotype 19F (amino acid identity, 57%; coverage, 96%)
cps8K	TIGR04370: glyco_rpt_poly; Oligosaccharide repeat unit polymerase	Oligosaccharide repeat unit polymerase Wzy	Polymerization of repeat units	Similar to oligosaccharide repeat unit polymerase Wzy of *S. pneumoniae* serotype 19F (amino acid identity, 54%; coverage, 94%)
cps8L	COG0381: WecB; UDP-N-acetylglucosamine 2-epimerase	UDP-N-acetylglucosamine-2-epimerase	ManNAc synthesis	Similar to UDP-N-acetylglucosamine-2-epimerase MnaA of *S. pneumoniae* serotype 19F (amino acid identity, 73%; coverage, 98%)
cps8M	cl09326: MATE_like super family; Multidrug and toxic compound extrusion family and similar proteins	Flippase Wzx	Transportation of repeat units	Similar to flippase Wzx of *S. pneumoniae* serotype 19F (amino acid identity, 50%; coverage, 93%)
cps8N	cl27287: GLF super family; UDP-galactopyranose mutase	UDP-galactopyranose mutase	Unknown	

cps8O	COG0431: SsuE; NAD(P)H-dependent FMN reductase	NAD(P)H-dependent FMN reductase	Unknown
cps8P	smart00347: HTH_MARR; Helix_turn_helix multiple antibiotic resistance protein	MarR family transcriptional regulator	Unknown

Figures

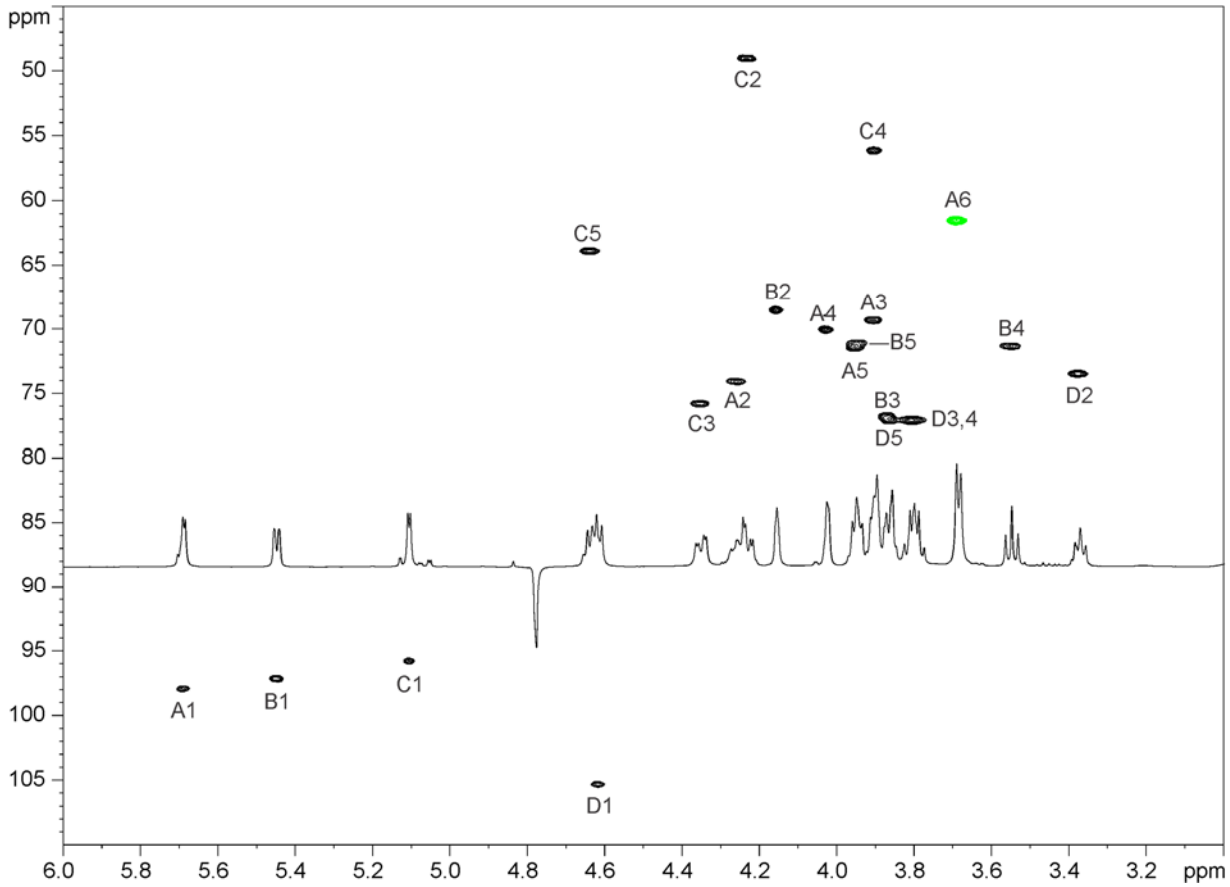


Figure 1. Enlargement of the ^{1}H-^{13}C HSQC and ^{1}H spectra of the *S. suis* serotype 7 capsular polysaccharide (25°C, 600 MHz).

Minor signals are from the hydrolyzed polymer.

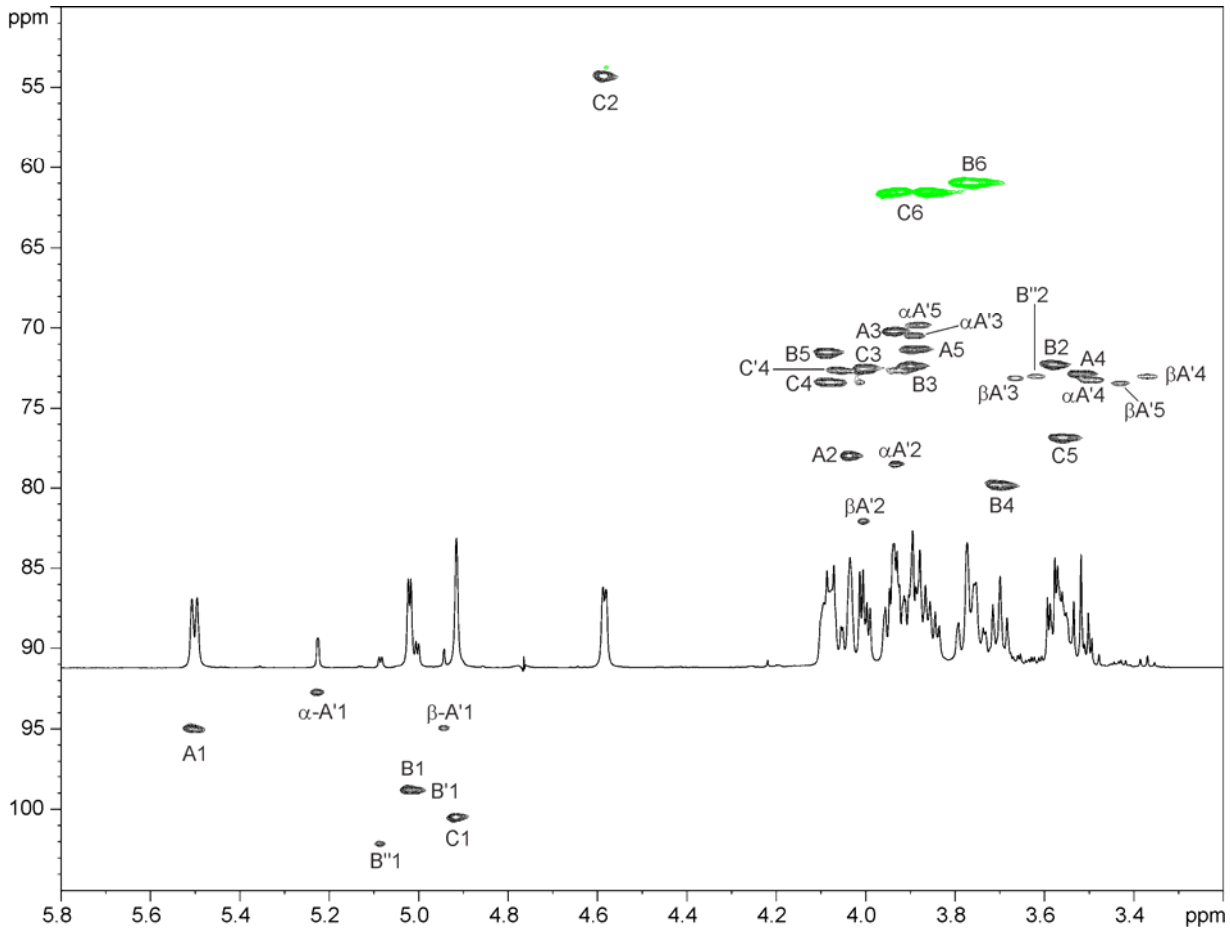


Figure 2. Enlargement of the ^{1}H-^{13}C HSQC and ^{1}H spectra of the *S. suis* serotype 8 capsular polysaccharide (25°C, 600 MHz).

Residues labeled αA', βA' are at the reducing end. B' is B linked to αA'; B'' is B linked to βA'. C' is the residue C at the non-reducing end.

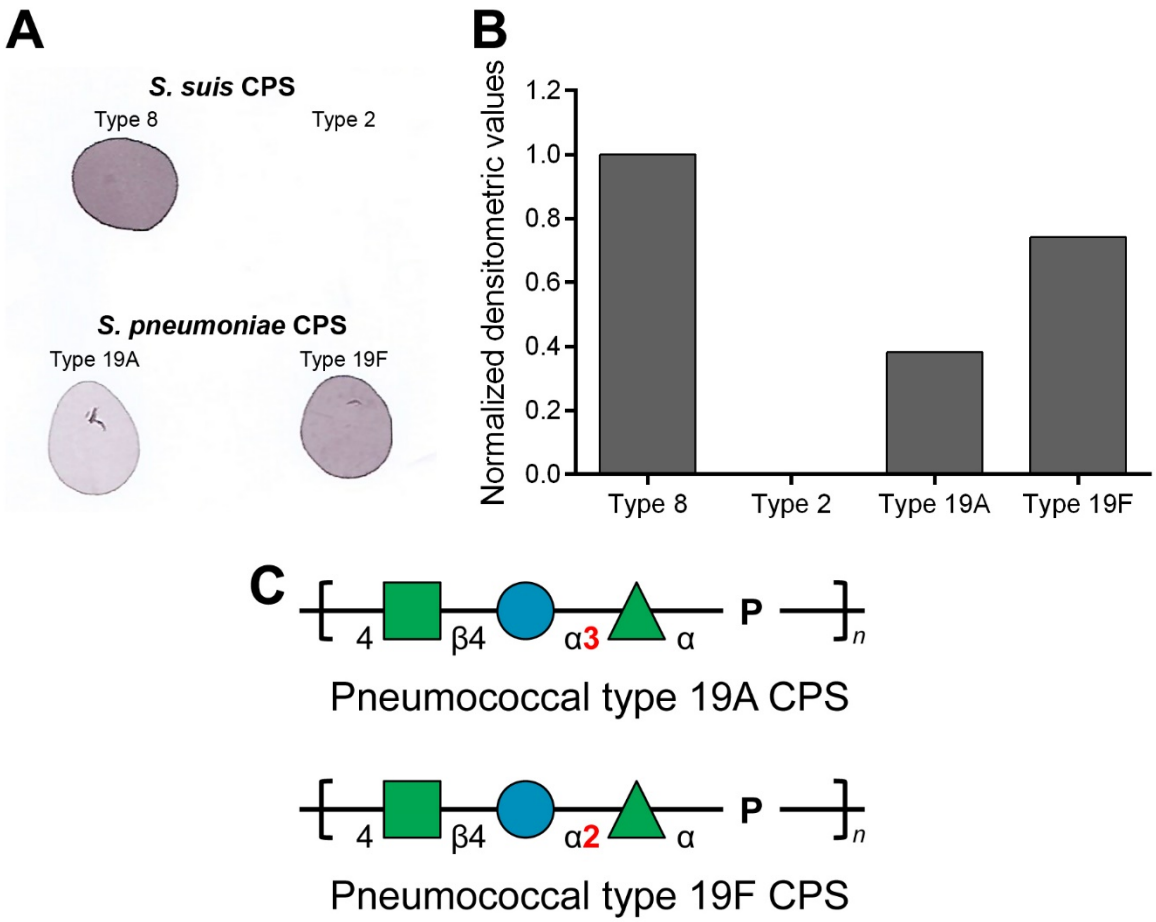


Figure 3. Serological cross-reactions of *S. suis* serotype 8 with pneumococcal types 19A and 19F.

(A) Dot-blot analysis with 10 µl (10 µg) of purified CPSs from pneumococcal types 19A and 19F using anti-*S. suis* serotype 8 serum. Purified CPSs of *S. suis* serotypes 8 and 2 were used as positive and negative controls, respectively. (B) Normalized integration values obtained by densitometry analysis were calculated. (C) Reported structures for the capsular polysaccharide repeating units of pneumococcal types 19A (43) and 19F (44). The difference in the position of the α-L-Rha-1-*P* between the two structures is shown in bold red. Monosaccharide symbols follow the SNFG (Symbol Nomenclature for Glycans) system (45).

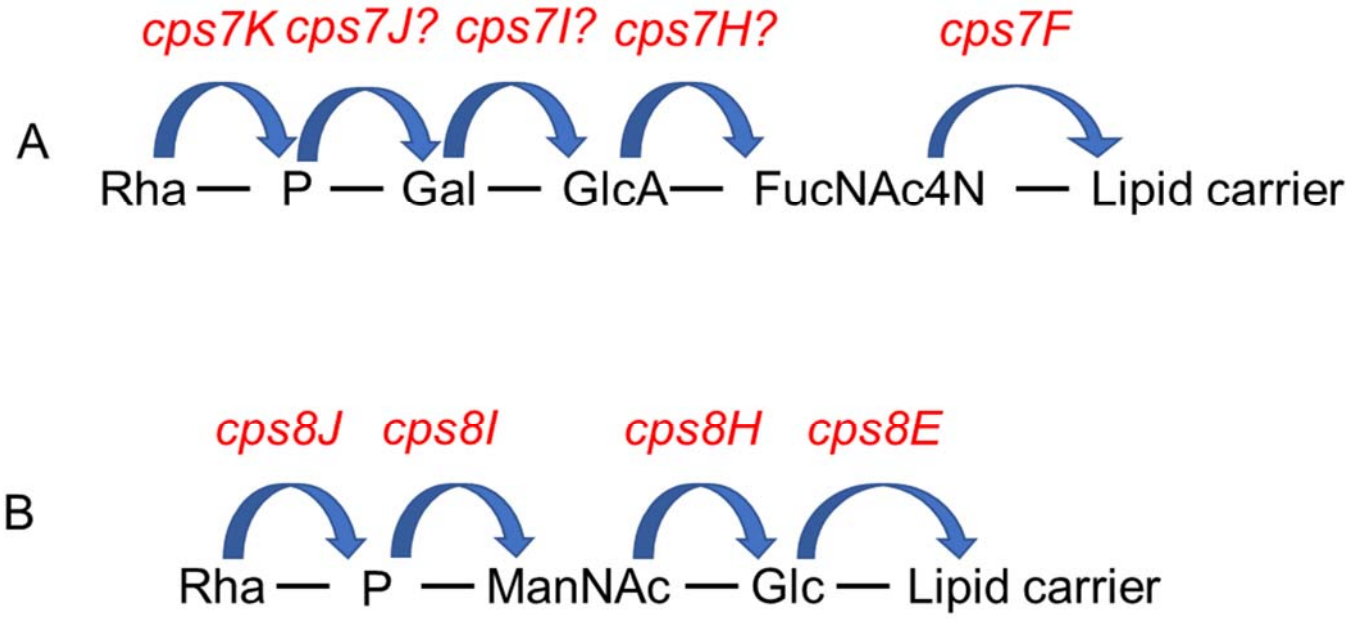


Figure 4. Tentative correlation between structure and genes encoding putative transferases responsible for the biosynthesis of *S. suis* serotypes 7 (A) and 8 (B) capsular polysaccharides (CPS).

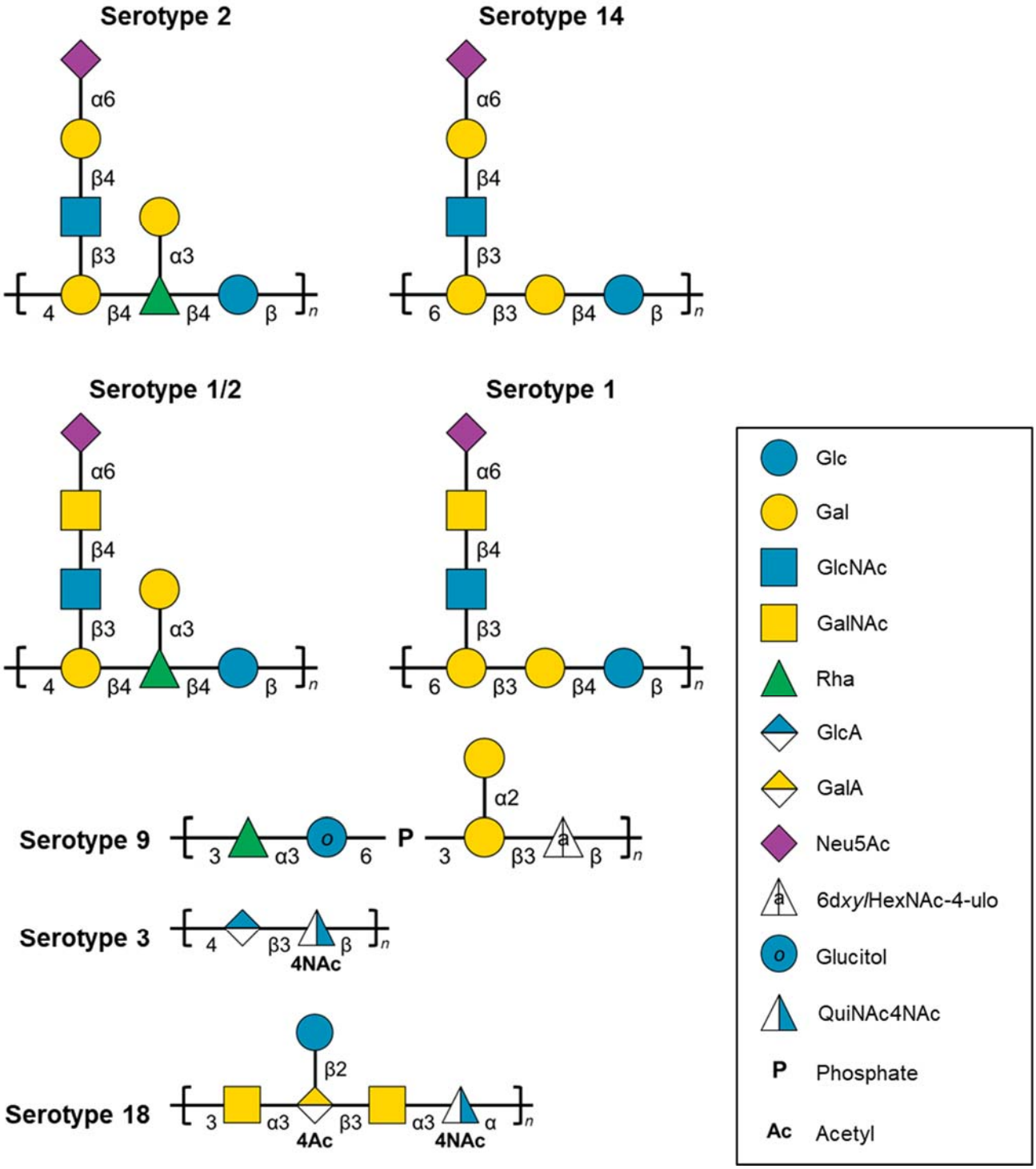


Figure S1. Reported structures for the known capsular polysaccharide repeating units of *S. suis* serotypes 2 (13), 14 (14), 1 & 1/2 (15), 9 (16), and 3 & 18(17).

Monosaccharide symbols follow the SNFG (Symbol Nomenclature for Glycans) system (45).

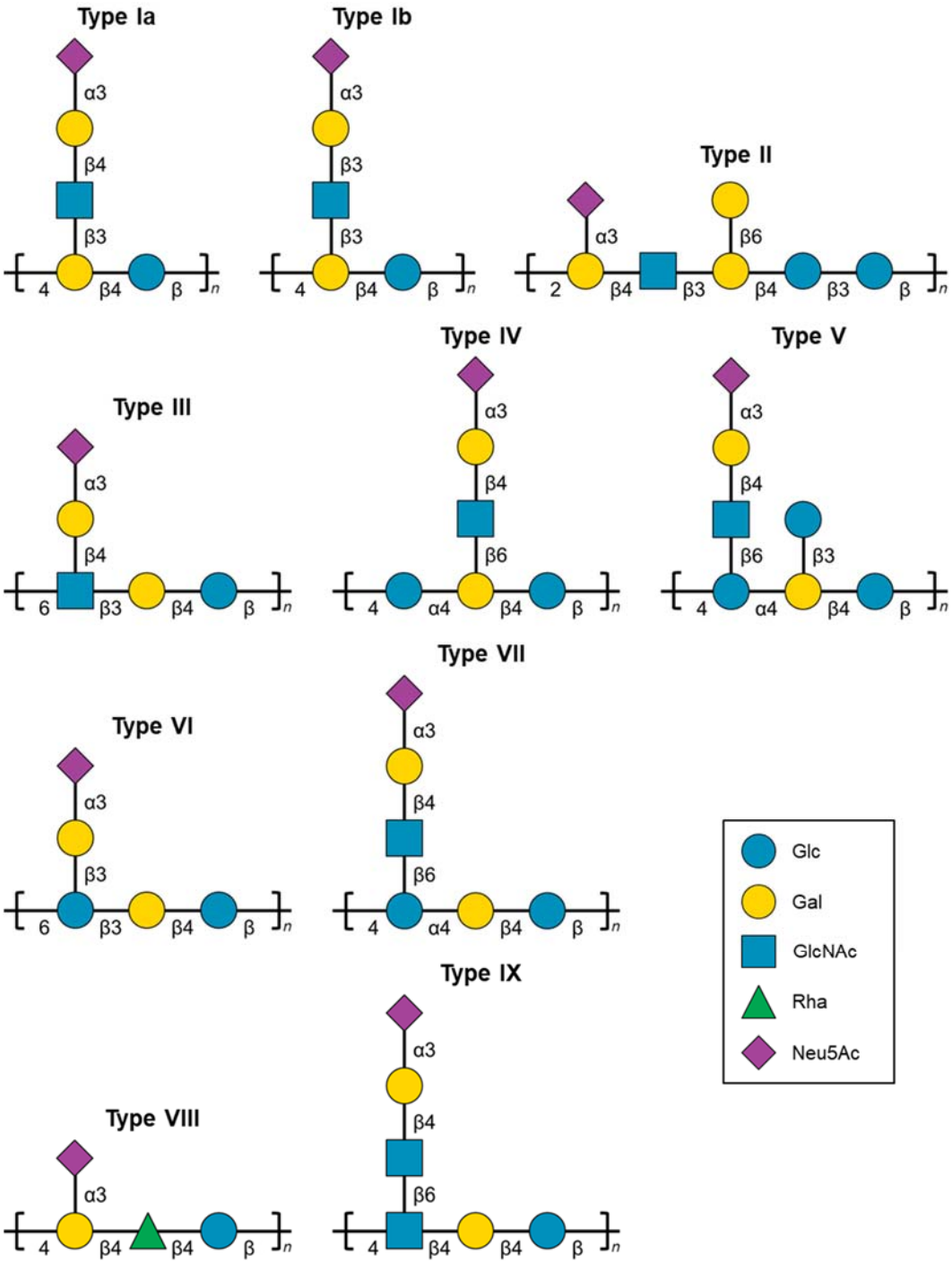


Figure S2. Reported structures for the capsular polysaccharide repeating units of Group B *Streptococcus* types Ia & Ib (46), II (47), III (48, 49), IV (50), V (51), VI (52), VII (53), VIII (54), and IX (55).

Monosaccharide symbols follow the SNFG (Symbol Nomenclature for Glycans) system (45).

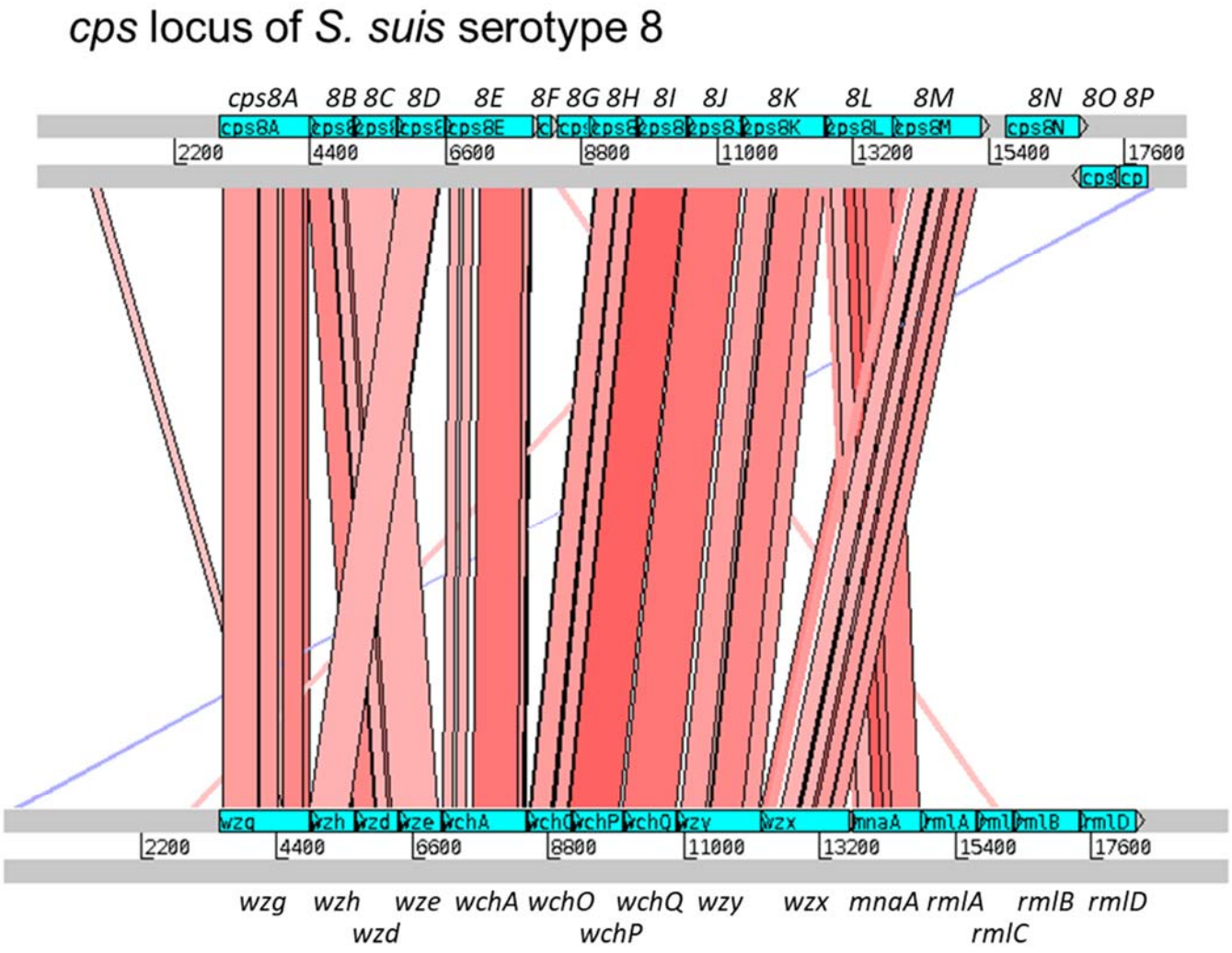


Figure S3. Comparison of *cps* gene clusters between *S. suis* 14636 (serotype 8) and *S. pneumoniae* 485/61 (serotype 19F) (GenBank accession nos. BR001005 and CR931678, respectively).

Pairwise TBLASTX comparison data (bit-scores above 100) was visualized by Artemis Comparison Tool (56).

ARTICLE IV

Comparative study of immunogenic properties of purified capsular polysaccharides from *Streptococcus suis* serotypes 3, 7, 8, and 9: the serotype 3 polysaccharide induces an opsonizing IgG response

---

Guillaume Goyette-Desjardins [1,3], Jean-Philippe Auger [1,3], Dominic Dolbec [1,3], Marie-Rose Van Calsteren [1,2], Marcelo Gottschalk [1,3], and Mariela Segura [1,3].

[1] Swine and Poultry Infectious Diseases Research Centre and Research Group on Infectious Diseases in Production Animals, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, Quebec, Canada

[2] Saint-Hyacinthe Research and Development Centre, Agriculture and Agri-Food Canada, Saint-Hyacinthe, Quebec, Canada

[3] Canadian Glycomics Network (GlycoNet), University of Alberta, Edmonton, Alberta, Canada.

Manuscrit en préparation pour soumission dans *Infection and Immunity.*

Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai participé activement à la conception de l'étude, à la réalisation des expériences (90%), à l'analyse des résultats et à l'écriture de l'article.

Abstract

Streptococcus suis is an encapsulated bacterium and one of the most important swine pathogens and a zoonotic agent for which no effective vaccine exists. Bacterial capsular polysaccharides (CPSs) are poorly immunogenic, but anti-CPS antibodies are essential to the host defense against encapsulated bacteria. In addition to the previously known serotypes 2 and 14, that are non-immunogenic, we have recently purified and described the CPS structures for serotypes 1, 1/2, 3, 7, 8, and 9. Here, we aimed to elucidate how these new structurally diversified CPSs interact with the immune system to generate anti-CPS antibody responses. CPS-stimulated dendritic cells produced significant levels of C–C motif chemokine ligand (CCL) 3 partially via Toll-like receptor (TLR) 2- and myeloid differentiation factor 88-dependent pathways, and produced CCL2 via TLR-independent mechanisms. Mice immunized with purified serotype 3 CPS adjuvanted with TiterMax Gold® produced an opsonizing IgG response, whereras other CPSs or adjuvants were negative. Mice hyperimmunized with either heat-killed *S. suis* serotypes 3 or 9 produced anti-CPS type 1 IgGs, whereas those hyperimmunized with either serotypes 7 or 8 no significant anti-CPS titers. Also, mice infected with sublethal doses of *S. suis* serotype 3 produced primary anti-CPS IgM and IgG responses, of which only IgM was boosted after a secondary infection. In contrast, mice infected with sublethal doses of *S. suis* serotype 9 produced weak anti-CPS IgM and IgG responses following a secondary infection. This study provides important information on the divergent evolution of CPS serotypes with highly different structural and/or biochemical properties within *S. suis* and their interaction with the immune system.

Introduction

Streptococcus suis is an encapsulated Gram-positive bacterium and one of the most important causes of bacterial infection and death in post-weaned piglets. This infection causes important economic losses to the swine industry and raises concerns about animal welfare. Striking manifestations of the disease in pigs are septicemia and meningitis, but other clinical manifestations can also be present as endocarditis and arthritis. *S. suis* is mainly localized in the upper respiratory tract of pigs, more particularly the tonsils and nasal cavities, and it may be acquired from the sows or through piglet-to-piglet transmission. Almost 100% of pig farms worldwide have carrier animals, although only a limited number of pigs develop clinical disease in the presence of preventive medication (1). However, recent implementation of compulsory reduction in the use of antibiotics in livestock production has led to a worriedly increase of *S. suis* clinical cases in swine productions. *S. suis* is also an emerging zoonotic agent, being responsible for meningitis, septic shock, and other less common infections usually related to generalized septicemia. This zoonosis is of particular importance worldwide to people in close contact with infected pigs and/or pork-derived products, especially in some Asian countries where the general population is at risk, since many cases of infection have been reported after ingestion of contaminated raw pork products (2, 3).

Cell-associated capsular polysaccharide (CPS) is considered to be one of *S. suis* most important virulence factors, a feature in agreement with other encapsulated pathogens such as *Streptococcus pneumoniae* and Group B *Streptococcus* (GBS) (4, 5). In fact, the originally described 35 serotypes of *S. suis* are based upon CPS antigenicity. Globally, the top 10 predominant serotypes in *S. suis* isolates reported between 2002 and 2013 from clinical cases in pigs are, in decreasing order, serotypes 2, 9, 3, 1/2, 8, 7, 4, 22, 5, and 1 (3). In humans, while the vast majority of infections are caused by serotypes 2 and 14, isolated cases caused by serotypes 1 and 9 have also been reported (3, 6, 7).

It is well known that the thick surface-associated CPS confers protection to *S. suis* against the immune system, notably by resisting phagocytosis (5, 8). Thus, as with other encapsulated pathogens such as *S. pneumoniae*, *Neisseria meningitidis*, *Haemophilus*

influenzae, and GBS, antibodies directed against the CPS are highly opsonizing and protective (9-11).

Dendritic cells (DCs), the most powerful antigen-presenting cells (APCs), express a wide variety of pattern recognition receptors (PRRs) that enable them to detect the presence of several pathogens through the recognition of pathogen-associated molecular patterns (PAMPs). Among these PRRs, Toll-like receptors (TLRs) are important for the initiation of the immune response as well as the shaping of adaptive immunity (12). Adaptive immune responses are strongly influenced by the interactions between DCs and pathogens, notably by the release of cytokines (13). However, due to their carbohydrate nature, bacterial CPSs are generally considered poorly immunogenic, since they are unable to recruit T cell help for B cell functions, classifying them as T cell-independent (TI) antigens (14). While several *in vitro* studies have demonstrated the ability of bacterial CPSs to interact with APCs, resulting in the production of cytokines and chemokines (15-19), others have linked this production with the presence of bacterial contaminants, such as TLR ligands, in purified CPS preparations (14, 20, 21). The adaptor molecule myeloid differentiation factor 88 (MyD88), which is involved in intracellular events downstream of TLR signaling, and TLR2 have been suspected to be involved in the interactions of bacterial CPSs with APCs leading to effective antibody responses (14, 16, 19, 20).

The repeating unit structure for *S. suis* serotype 2 CPS has been reported in 2010, followed by that for serotype 14 in 2013 (Fig. 1) (22, 23). Working with those two serotypes, Calzas *et al.* have first demonstrated that stimulation of DCs with purified CPSs induced no cytokine production but led to an important production of C–C motif chemokine ligand (CCL) 2 and CCL3 (24). Secondly, they have demonstrated a very low or undetectable primary anti-CPS IgM response with only a moderate boost in IgM and no isotype switching as features of the antibody response following mouse infections (25, 26). Thirdly, they have been unable to measure anti-CPS antibody responses *in vivo* or anti-CPS antibody-secreting cells *ex vivo* following mouse immunization with purified *S. suis* serotypes 2 or 14 CPSs (26).

Since then, our team, which is the only one to report and expand the known CPS structures of *S. suis*, has published complete structural determination for the CPSs of serotypes 1 & 1/2 (27), serotype 9 (28), serotypes 3 & 18 (29), and serotypes 7 & 8 (30). Serotypes 2, 14, 1, and 1/2 CPSs are structurally related, as they all possess an α2,6-linked sialic acid and are of similar compositions: a key feature of these four serotypes is that serotypes 2 and 14 both possess a β-galactose (Gal) in their side chain that is replaced by *N*-acetylgalactosamine (GalNAc) in serotypes 1 and 1/2 (Fig. 1) (27). This substitution has been linked to a single amino acid polymorphism in the glycosyltransferase CpsK that defines the enzyme substrate predilection for Gal or GalNAc and therefore determines CPS composition, structure, and strain serotype (31, 32). Thus, our first hypothesis is that the CPSs of serotypes 1 and 1/2 are as non-immunogenic as those of serotypes 2 and 14 due to their structural similarity. CPSs of serotypes 3, 7, 8, and 9, on the other hand, are structurally well diversified among themselves and from those of the previous four serotypes: they do not possess sialic acid, some contain phosphates in their backbone, and others contain unusual sugars such as di-*N*-acetylbacillosamine (QuiNAc4NAc), *N*-acetyl-4-aminofucosamine (Fuc2NAc4N), glucitol, and a 4-keto sugar (6d*xyl*HexNAc-4-ulo) (Fig. 1). Additionally, the CPS of *S. suis* serotype 8 has been shown to be identical to that of *Streptococcus pneumoniae* serotype 19F (Fig. 2) (30, 33). Pneumococcal serotype 19F CPS is considered to be immunogenic, as it is included in the 23-valent pneumococcal polysaccharide vaccine (34). Accordingly, our second hypothesis is that, due to their structural differences, the CPSs of serotypes 3, 7, 8, and 9 have the potential to differently modulate DC activation and the subsequent humoral immune response. Therefore, using purified CPSs, we aimed to study their ability to stimulate antigen-presenting APCs *in vitro* and to perform *in vivo* immunogenicity studies with the newly described serotypes 1, 1/2, 3, 7, 8, and 9 of *S. suis*.

Material and Methods

Bacterial strains and culture conditions

Bacterial strains that were used in this study are listed in Table 1. Isolated colonies on sheep blood agar plates were inoculated in 5 ml of Todd-Hewitt broth (THB) (Oxoid, Nepean, ON, Canada) and incubated for 8 h in a water bath at 37°C with agitation at 120 rpm. Working cultures were prepared by transferring 10 µl of 8-h cultures diluted 1:1,000 with phosphate-

buffered saline (PBS) into 30 ml of THB, which was incubated for 16 h. Bacteria were washed three times and resuspended in PBS to obtain a concentration between 1×10^8 to 1×10^9 CFU/ml. Heat-killed bacterial cultures were obtained as previously described (35). Briefly, overnight cultures were washed three times with PBS and then resuspended in 30 ml of fresh PBS. A sample was taken to perform bacterial counts on Todd-Hewitt agar (THA). Bacteria were immediately killed by incubating at 60°C for 45 min and then were cooled on ice. Bacterial killing was confirmed by absence of growth on blood agar for 48 h.

A CPS-switched 2to3 mutant derived from the same *S. suis* serotype 2 strain was also used for the sublethal infections (Table 1). This mutant has been obtained by genetic excision in the receiver strain of the whole *cps2* locus genes (responsible for CPS expression) followed by replacement with the whole *cps3* locus genes obtained from a serotype 3 donor strain (Dr. M. Okura, unpublished data). Verification of this mutant by whole-genome sequencing, transmission electron microscopy, serology, and NMR analyses of its purified CPS all confirmed its phenotype as a CPS 3-expressing well-encapsulated mutant (Dr. M. Okura, unpublished data). While expressing CPS 3 as its capsule, the subcapsular antigens of this mutant are from a phenotypically very different serotype 2 strain.

S. suis capsular polysaccharides – production and purification

The following methods for CPS production and purification were adapted from previous publications (22, 27-30). Briefly, 6 L of fresh THB was inoculated with the appropriate strain and incubated overnight. For more information on the reference strains used for CPS production, refer to Table 1.

Depending on the sensibility of the specific CPS to hydrolysis, two methods of CPS extraction were used. For serotypes 2, 1, 1/2, and 3, the **autoclave method** was used (22, 27, 29). Briefly, bacterial cells from the 6-L culture were pelleted by centrifugation at 10,000 × *g* for 40 min, suspended by repeated pipetting in a buffer containing 33 mM phosphate and 145 mM NaCl pH 8.0, and chilled. The bacterial suspension was autoclaved at 121°C for 15 min. The supernatant containing the crude CPS was recovered by centrifugation at 9,000 × *g* for 50 min. For serotypes 7, 8, and 9, the **water method** was used (28-30). Briefly, the bacterial

pellet from the 6-L culture was resuspended in deionized water (ddH_2O). The bacteria were killed by heating as described above. The content of the tubes was freeze-dried for 72 h. Then, bacterial cells were stirred with ddH_2O (3 g of dry cells in 100 ml) overnight at room temperature. The supernatant containing the crude CPS was recovered by centrifugation at 9,000 × *g* for 50 min.

The crude CPSs were further purified from the extracted material by solvent extraction with chloroform to remove lipids, precipitation with 25% (v/v) ethanol and 0.1 M $CaCl_2$ to remove nucleic acids, precipitation of CPS and other macromolecules with 80% (v/v) ethanol, dialysis, and freeze-drying. The final purification step to remove proteins was performed by gel filtration chromatography as previously described (29). Briefly, a XK-26/100 column packed with Sephacryl S-400 HR (GE Healthcare Life Sciences, Uppsala, Sweden) was used and eluted with 50 mM NH_4HCO_3 (for serotypes 2, 1, 1/2, and 3) or with 50 mM NaCl (for serotypes 7, 8, and 9) at a flow rate of 1.3 mL/min, using an ÄKTA Purifier 10 system (GE Healthcare Life Sciences), including a UV-900 Monitor, and equipped with a Knauer Smartline 2300 RI Detector (Knauer, Berlin, Germany) connected to the system via an AD-900 Analog/Digital Converter (GE Healthcare Life Sciences). Fractions giving a positive RI signal but no absorption at 280 and 254 nm were pooled and freeze-dried. Dot-blot confirmation of CPS-containing fractions with reference antisera has already been performed previously (22, 27-30). To desalt, the purified material was dissolved in water, dialyzed against ddH_2O for 24 h at 4°C, and finally freeze-dried.

S. pneumoniae capsular polysaccharide

Purified pneumococcal polysaccharide of type 19F was manufactured by Pfizer and purchased through the American Type Culture Collection (ATCC 84-X®).

CPS quality controls

Each CPS was subjected to rigorous quality control tests as previously described (24) on 1-mg/ml samples. Nucleic acids were quantified using an ND 1000 spectrometer (Nanodrop). The absorbance was measured at 230 and 260 nm on CPS samples. Calculations were done with the Nanodrop software. According to the manufacturer, results are

reproducible between 2 and 100 ng/µl. Proteins were quantified on CPS samples using the Modified Lowry Protein Assay Kit (Thermo Fisher Scientific, Saint-Laurent, Quebec, Canada) in a 96-well microplate according to the manufacturer instructions. The calculated limit of detection ($P \leq 0.05$) was 4 µg/ml for the Modified Lowry assay. Proteins were also quantified on CPS samples using the NanoOrange Protein Quantitation Kit (Molecular Probes, Eugene, OR) according to manufacturer instructions by heating samples into microtubes, followed by transfer to a 96-well microplate for fluorescence reading. The calculated limit of detection ($P \leq 0.05$) was 5 µg/ml for the NanoOrange assay.

Mouse strains

Mice originating from Jackson Laboratory (Bar Harbor, ME), including wild-type (WT) C57BL/6J, MyD88$^{-/-}$ (B6.129P2(SJL)-*Myd88*^{tm1Defr}/J), and TLR2$^{-/-}$ (B6.129-*Tlr2*^{tmlKir}/J) mice, were used to generate bone marrow-derived DCs. C57BL/6 mice from Charles River (Wilmington, MA) were used to perform the immunizations and infections. All experiments involving mice were carried out in accordance with the recommendations of the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The protocols and procedures were approved by the Animal Welfare Committee of the University of Montreal (protocol numbers rech-1399 and rech-1523).

Generation of bone marrow-derived DCs

Bone marrow-derived DCs were produced according to a previously described technique (24, 36) and cultured in complete cell culture medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 U/ml penicillin-streptomycin, and 20 µg/ml gentamicin. All reagents were from Gibco (Invitrogen, Burlington, ON, Canada). Cell purity was routinely at ≥ 86–90% CD11c^{+high} F4/80$^{-/dim}$ cells as determined by fluorescence-activated cell sorter analysis (24, 37).

In vitro DC stimulation assay

DCs were resuspended at 10^6 cells/ml in complete medium and stimulated with CPS (final concentration of 200 µg/ml) essentially as described in Calzas *et al.* (24). CPS samples were prepared from 2-mg/ml stock solutions and were treated with polymyxin B sulfate (final concentration of 20 µg/ml; Sigma-Aldrich, Oakville, ON, Canada) 1 h prior to activation to neutralize any possible endotoxin contamination.

After 24 h, supernatants were collected for cytokine quantification by ELISA. Cells stimulated with 1 µg/ml purified *Escherichia coli* O127:B8 lipopolysaccharide (LPS) (Sigma-Aldrich) served as a positive control for cytokine production. Non-stimulated cells served as a negative control. All solutions were tested for the absence of endotoxin by use of a *Limulus* amebocyte lysate gel-clotting test (Pyrotell; ACC, East Falmouth, MA).

Cytokine quantification by enzyme-linked immunosorbent assay (ELISA)

Levels of interleukin (IL)-6, tumor necrosis factor (TNF), CCL2, and CCL3 in cell culture supernatants were measured by sandwich ELISAs using pair-matched antibodies from R&D Systems (Minneapolis, MN), performed according to the manufacturer's recommendations.

Mouse immunization studies

Five-week-old C57BL/6 female mice were immunized subcutaneously with a dose of 25 µg of purified CPS in 0.1 ml of PBS on day 0 and boosted on day 21 as previously reported (11). In a first experiment aimed to evaluate the immunogenicity of the CPSs from the different serotypes, 5 groups of mice ($N = 10$) received 25 µg of either *S. suis* serotype 2 CPS (CPS 2), CPS 3, CPS 7, CPS 8, or CPS 9 dissolved in PBS and emulsified 1:1 (v/v) with TiterMax Gold® (CytRx Corporation, Norcross, GA) as the adjuvant. One placebo group of mice ($N = 10$) received PBS adjuvanted with TiterMax Gold® as described above. In a second experiment aimed to compare different adjuvants with CPS 3, three groups of mice ($N = 10$) received 25 µg of CPS 3 either dissolved in PBS (without adjuvant) or adjuvanted with either 1:1 (v/v) with Alhydrogel 2%® (Brenntag Biosector, Frederikssund, Denmark) or with 20 µg of Quil-A® (Brenntag Biosector). Three placebo groups of mice ($N = 10$) were included: the

first received PBS only (no adjuvant), the second received PBS adjuvanted with Alhydrogel 2%®, and the third received PBS adjuvanted with Quil-A® as described above. In a third experiment aimed to evaluate the immunogenicity of phenol-extracted CPS 3 (described below), two groups of mice (N = 10) received 25 µg of either native or phenol-extracted CPS 3 adjuvanted with TiterMax Gold® (see above). One placebo group of mice (N = 5) which received PBS adjuvanted with TiterMax Gold® was also included.

In the first experiment to follow the kinetics of the antibody responses, mice were bled (10 µl) weekly on days −1, 7, 14, 20, 28, 35, and 41 post-immunization by the tail vein. In the two other experiments, mice were bled (10 µl) on days −1, 20, and 41 post-immunization by the tail vein to follow antibody responses. Diluted blood was directly used in the ELISA as described below. At day 42 post-immunization, mice were humanely euthanized and sera collected and frozen at −80°C for ELISA Ig titration and isotyping analyses and for opsonophagocytosis assay (OPA) (see below).

Control antisera – mouse hyperimmunization

Hyperimmune mice (N = 3–4) were obtained by repeated immunization of 5-week-old female C57BL/6 mice with 1×10^9 CFU/ml heat-killed *S. suis* in THB by intraperitoneal injection weekly during 4 to 8 weeks. For more information on the strains used for mouse hyperimmunization, refer to Table 1. Two weeks after the last injection, sera were collected, pooled by serotype, aliquoted, and stored at −80°C.

Measurement of antibodies against S. suis CPSs and whole bacteria

The following methods for coating of microplates and the measurement of antibodies by ELISA were taken or adapted from previous publications (11, 25, 38). For coating of ELISA plates with CPS 2, CPS 3, and CPS 9, CPSs were diluted to 2 µg/ml in 0.1 M $NaCO_3$ (pH 9.6), then 100 µl were added to wells of a 96-well Polysorp microplate (Nunc-Immuno; Canadawide Scientific, Toronto, Ontario, Canada). For coating with CPS 7 and CPS 8, the CPSs were diluted to 10 and 5 µg/ml, respectively, then 100 µl were added to wells of a 96-well EIA/RIA microplate (medium binding; Thermo Fisher Scientific). CPS-coated plates were left overnight at 4°C. For coating of ELISA plates with whole bacteria, bacterial cultures

were prepared as described above and resuspended in ddH_2O to an OD_{600} of 0.500–0.550, which corresponds to approximately 1×10^8 CFU/ml. For more information on the strains used for ELISA plate coating, refer to Table 1. To the wells of a 96-well Polysorp microplate was added 100 µl of the bacterial suspension, and left to air-dry at room temperature for 48 h. Then, 50 µl of methanol was added to each well and left to evaporate at room temperature for a few hours. When completely dry, plates were stored at room temperature.

Plates were washed with PBS containing 0.05% (v/v) Tween 20 (PBST) and blocked by treatment with 300 µl of PBS containing 1% (w/v) bovine serum albumin (HyClone, Logan, UT) for 1 h. After washing, 100 µl of mouse blood or serum samples diluted in PBST were added to the wells and left for 1 h. After washing, the plates were incubated for 1 h with 100 µl of an horseradish peroxidase (HRP)-conjugated isotype specific antibody diluted in PBST as described below. The enzyme reaction was developed by addition of 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB; 100 µl) (Invitrogen) and stopped by addition of 50 µl of 0.5 M H_2SO_4, and the absorbance was read at 450 nm with an ELISA plate reader.

To follow the kinetics of total Ig [IgG + IgM] antibody responses to CPS, mouse blood collected from the tail vein was diluted 1:100. Dilution optimization had previously been conducted (data not shown). HRP-conjugated goat anti-mouse total Ig [IgG + IgM] (H+L) at a dilution of 1:2,500 (Jackson ImmunoResearch, West Grove, PA) was used as a detection antibody.

To perform the titration of mouse Ig isotypes, day-42 serum was serially diluted (2-fold) in PBST, and antibodies were detected using either HRP-conjugated goat anti-mouse total Ig [IgG + IgM] as mentioned above, goat anti-IgG (Fcγ fragment specific; Jackson Immunoresearch), goat anti-IgM diluted 1:1000, goat anti-IgG1, goat anti-IgG2b, goat anti-IgG2c, or goat anti-IgG3 diluted 1:400 (Southern Biotech, Birmingham, AL). For mouse serum titration, the reciprocal of the last serum dilution that resulted in an optical density at 450 nm (OD_{450}) equal to or lower than 0.2 (as a pre-established cutoff for comparison purposes) was considered the titer of that serum. For representation purposes, negative titers (less than or equal to the cutoff) were given an arbitrary titer value of 50.

To control inter-plate variations, an internal reference positive control was added to each plate. For titration of mouse antibodies, this control was a pool of sera from hyperimmunized mice (produced as described above). Reaction in TMB was stopped when an OD_{450} of 1.0 was obtained for the positive internal control. Optimal coating conditions for the anti-CPS and anti-*S. suis* ELISAs, optimal dilutions for the positive internal control sera, and the HRP-conjugated anti-mouse antibodies were determined during preliminary standardizations.

Opsonophagocytosis assay (OPA) for serotype 3

Instead of using a cell line or a single cell type, the OPA was standardized using whole blood from naive mice (11, 39). This model considers all blood leukocytes and thus represents a more realistic model of the complex interactions between all immune cells and the bacteria during a systemic infection, as is the case for *S. suis*.

Murine whole blood OPAs were performed as previously described (11, 39). Blood was collected by intracardiac puncture from naïve C57BL/6 mice, treated with sodium heparin, and then diluted to obtain 6.25×10^6 leukocytes/ml in complete cell culture medium. All blood preparations were kept at room temperature. Using washed bacterial cultures of *S. suis* serotype 3 strain 4961 (Table 1) grown as described above, final bacterial suspensions were prepared in complete cell culture medium to obtain a concentration of 1.25×10^6 CFU/ml. The number of CFU/ml in the final suspension was determined by plating samples onto THA. All bacterial preparations were kept on ice.

Diluted whole blood (5×10^5 leukocytes) was mixed with 5×10^4 CFU of *S. suis* (multiplicity of infection [MOI] of 0.1) and 40% (v/v) of serum from naive or vaccinated mice in a microtube to a final volume of 0.2 ml. The tube tops were pierced using a sterile 25-gauge needle, and then the microtubes were incubated for 2 h at 37°C with 5% CO_2, with gentle manual agitation every 20 min. After incubation, viable bacterial counts were performed on THA. Tubes with addition of naive rabbit serum or rabbit anti-*S. suis* type 3 serum (40) were used as negative and positive controls, respectively. The bacterial killing percentage was

determined using the following formula: percentage of bacteria killed = [1 − (bacteria recovered from sample tubes/bacteria recovered from negative-control tubes with naive mouse sera)] × 100. Final OPA conditions were selected based on several pretrials using different incubation times and MOIs (data not shown).

Phenol extraction of S. suis serotype 3 CPS (CPS 3)

Phenol extraction of CPS 3 was conducted as described by Sen *et al.* (20) with some modifications. Native CPS 3 was dissolved in water containing 0.2% of triethanolamine (TEA) to a final CPS concentration of 5 mg/ml. Sodium deoxycholate was omitted, as it caused CPS 3 to precipitate. Water-saturated phenol (0.5 ml) was added, and the tube was vortexed intermittently for 5 min. The solution was left standing for 5 min at room temperature for phase separation, then was placed on ice for an additional 5 min. The material was centrifuged for 2 min at 10,000 × *g* at 4°C. The upper aqueous layer was transferred to another tube. The phenol layer was re-extracted with 0.5 ml of water containing 0.2% TEA. The aqueous layers were pooled and re-extracted with 1 ml of water-saturated phenol. Pooled aqueous layers were adjusted to 80% ethanol containing 30 mM sodium acetate and left overnight at 4°C to precipitate. The material was then centrifuged for 10 min at 10,000 × *g* at 4°C. The precipitate was washed with 1 ml of ice-cold absolute ethanol and then air-dried. CPS 3 was redissolved in 1 ml of water and freeze-dried to be weighed subsequently.

Dot-blot

Dot-blot analyses were performed essentially as described previously by Van Calsteren *et al.* (27). Ten microliters (each at 1 mg/ml) of both native and phenol-extracted CPS 3 and of CPS 2 (used as negative control) were blotted on a PVDF Western blot membrane (Bio-Rad, Hercules, CA). The membrane was blocked for 1 h with a solution of Tris-buffered saline (TBS) containing 2% skim milk, followed by 2 h of incubation with rabbit anti-*S. suis* type 3 serum. The membrane was washed three times with TBS, and anti-rabbit HRP-conjugated antibody (Jackson Immunoresearch) was added for 1 h. The membrane was washed three times with TBS and revealed with a 4-chloro-1-naphthol solution (Sigma-Aldrich).

Nuclear magnetic resonance (NMR) spectroscopy

Both native and phenol-extracted CPS 3 were exchanged in 33 mM phosphate buffer pD 8.0 in D_2O (99.9 atom% D), freeze-dried, and dissolved in D_2O (99.96 atom% D). NMR spectra were acquired on CPS samples at concentrations of 0.6–0.7% at 11.75 T using a Bruker Avance 500 spectrometer equipped with a 5-mm triple resonance TBI probe with ^{1}H, ^{13}C, and ^{109}Ag–^{31}P channels at 60°C using standard Bruker pulse sequences at the Centre régional de résonance magnétique nucléaire (Department of Chemistry, University of Montreal). For conventional ^{1}H spectra, 32 K complex data points were acquired after a 30° pulse with a digital resolution of 0.18 Hz/point and processed off-line using SpinWorks version 4.2.8.0 (Copyright © Kirk Marat, home.cc.umanitoba.ca/~wolowiec/spinworks/) by exponential multiplication with a 0.2-Hz line broadening factor, zero filling, complex Fourier transform, phase correction, and fifth-order polynomial baseline correction. ^{1}H chemical shifts δ in ppm were referenced to internal deuterated 2,2-dimethyl-2-silapentane-5-sulfonate at δ 0 as recommended by Wishart *et al.* (41).

Experimental mouse sublethal infections

Six-week-old female C57BL/6 were injected intraperitoneally with sublethal doses of the *S. suis* strains employed (1×10^6 CFU for serotype 2, serotype 3, and CPS-switched 2to3 mutant; and 1×10^5 CFU for serotype 9). For more information on the strains used for experimental infections, refer to Table 1. These sublethal doses were chosen, as they produced little to no clinical signs and mortality in our infection model in order to follow the antibody response. For the primary infection model, groups of mice (N = 4–10) were infected on day 0 followed by serum collection on day 21. For the secondary infection model, groups of mice (N = 9–10) were infected on day 0, reinfected on day 21, then sera were collected on day 42. Placebo groups of mice (N = 5–10) were similarly injected once or twice with the vehicle solution (sterile THB). Infected animals were monitored at least twice daily for the first 7 days after each infection, then daily until the end of the study. Blood bacterial titers were assessed 24 h following the first injection on day 0 by collecting 5 µl of blood from the tail vein. Proper dilutions in PBS were plated onto THA.

Statistical analyses

Parametric data are expressed as means ± standard errors of the means (SEM) and were analyzed for significance using one-way analysis of variance (ANOVA) followed by the Tukey test. Non-parametric data are shown with the median and were analyzed for significance using either the Mann-Whitney Rank Sum Test (to compare 2 groups) or the Kruskall-Wallis ANOVA on ranks followed by the Dunn procedure (to compare 3 groups or more). Statistical analyses were performed using Systat SigmaPlot version 11.0.

Results

S. suis CPS purification and quality controls

In order to perform immunogenicity testing of purified CPSs of *S. suis* serotypes 2, 1, 1/2, 3, 7, 8, and 9, reference strains (Table 1) were used. Quality controls of polysaccharides were performed to ensure the absence of immunogenic contaminants that may bias our study (Table 2). Results revealed that less than 1% (w/w) of nucleic acids and proteins were detected in the purified CPS samples, with the only exception being CPS 9 which showed a striking protein content of 6.7% (w/w). One possible explanation for that unusual result is that the Modified Lowry Assay is subject to interference by substances with reducing potential, such as the unique 4-keto sugar of CPS 9 (2-acetamido-2,6-dideoxy-β-D-*xylo*-hexopyranos-4-ulose). To test this hypothesis, the NanoOrange Assay was performed on selected samples, as this assay uses a hydrophobic fluorescent dye and possesses a sensitivity similar to the Modified Lowry Assay (Table 2). Using the NanoOrange Assay, no protein were found above the detection limit of 0.4% (w/w), confirming that CPS 9 is indeed free of protein, and also that the result obtained with the Modified Lowry Assay is due to an interference by its 4-keto sugar.

S. suis CPSs induce the release of chemokines by DCs

To study the ability of purified CPSs of *S. suis* to stimulate APCs *in vitro*, mouse bone marrow-derived DCs were chosen. Herein, following a 24-h stimulation of DCs with 200 µg/ml for each *S. suis* CPS, the levels of two pro-inflammatory cytokines in the supernatant, namely IL-6 and TNF, were measured. No significant difference in cytokine production was

observed between DCs incubated with the different CPSs and those incubated with medium alone ($P > 0.05$) (Fig. 3). For reference, purified *Streptococcus pneumoniae* serotype 19F CPS preparation for vaccine production was purchased and used to stimulate DCs in the same manner. As shown in Fig. 3, pneumococcal CPS 19F induced strong production of both IL-6 and TNF.

In contrast to this pro-inflammatory cytokines, all *S. suis* and *S. pneumoniae* CPSs at 200 µg/ml induced significant release of the chemokines CCL2 and CCL3 after incubation with DCs (Fig. 4), we also investigated the involvement of PRRs in chemokine release by CPS-stimulated DCs of CCL2 via MyD88-independent and of CCL3 via TLR2-dependent and MyD88-dependent pathways on comparison of WT DCs with either $TLR2^{-/-}$ or $MyD88^{-/-}$ DCs. Results showed that no significant difference in CCL2 production was found (Fig. 4A). This indicates that CCL2 production by DCs is induced through a different pathway than the TLRs. However, CCL3 production was significantly reduced in $TLR2^{-/-}$ DCs and further reduced in $MyD88^{-/-}$ DCs, although not abrogated (Fig. 4B). This indicates that CCL3 production by DCs is partially dependent upon TLR2 and MyD88 signaling, but also that at least another pathway is involved in CPS-mediated activation of DCs. Additionally, CCL2 and CCL3 production by CPS-stimulated WT DCs appears to be independent of the CPS structure, as levels for both chemokines were all similar amongst samples from *S. suis* and *S. pneumoniae* (Fig. 4).

In vivo immunogenicity studies with purified CPSs 3, 7, 8, and 9

Despite the absence of cytokine production and the lack of differences in chemokine production patterns after stimulation of APCs with widely different CPSs, the immunogenicity of those newly obtained purified CPSs was evaluated *in vivo*.

Native purified CPS 2 (included as control) or native CPSs 3, 7, 8, and 9 were emulsified with TiterMax Gold® and administered to mice on days 0 and 21. CPSs 2, 7, 8, and 9 failed to induce a significant anti-CPS response (Fig. 5). While CPS 2 is already known to be poorly immunogenic, additional control ELISAs were performed for CPSs 7, 8, and 9 to ensure that the lack of anti-CPS response was not due to problems with the coating (Suppl. Fig. 1). Firstly, ELISA plates coated with whole *S. suis* of either serotype 7, 8, or 9 were used

to validate a strong total Ig [IgG + IgM] anti-*S. suis* responses in sera from hyperimmunized mice (Suppl. Fig. 1A–C). Secondly, these control hyperimmune mouse sera showed no anti-CPS response for serotypes 7 and 8 (Suppl. Fig. 1D–E) but an anti-CPS response for serotype 9 (Suppl. Fig. 1F) using CPS-coated plates. Finally, sera collected at day 42 from mice immunized with purified CPSs 7, 8, and 9 showed no significant total Ig [IgG + IgM] anti-*S. suis* responses ($P > 0.05$ *vs.* placebo groups) using whole-bacteria-coated plates. The latter result further supports that these CPSs did not elicit antibodies in the immunized animals and that this lack of response was not related to coating technical issues (Suppl. Fig. 1G–I). Taken together, these data indicate that purified CPSs 7, 8, and 9 are not immunogenic in mice.

In contrast, mice immunized with the native purified CPS 3 produced a strong antibody response as early as 7 days after a single dose, followed by a slight increase in the response following a boost on day 21 (Fig. 5B). A stronger response following boosting but also antibody isotype switching are good indicators of immunogenicity. Therefore, titers of the different anti-CPS antibody isotypes were determined in mice immunized with 25 µg of CPS 3 adjuvanted with TiterMax Gold®. As shown in Fig. 6A, not only strong IgM titers but also significant high levels of type 1 IgG subclasses (IgG2b, IgG2c, and IgG3) were observed ($P \leq 0.05$ *vs.* placebo group), whereas no type 2 IgG subclass (IgG1) was detected ($P > 0.05$ *vs.* placebo group). Based on these observations, the protective capacity of sera from CPS 3-immunized mice was evaluated using an OPA test, a recognized correlate of immunity for encapsulated Gram-positive bacteria (42). As shown in Fig. 6B, sera from mice immunized with CPS 3 adjuvanted with TiterMax Gold® induced high bacterial killing levels ($P \leq 0.001$ *vs.* placebo group).

Adjuvant effect on in vivo immunogenicity of purified CPS 3

Results showed that mice receiving on days 0 and 21 the same dose as before (25 µg) of CPS 3 either non-adjuvanted (PBS only) or adjuvanted with 1:1 (v/v) with Alhydrogel 2%® (43, 44) or 20 µg of Quil-A® (45, 46) able to elicit anti-CPS 3 antibodies in the immunized mice (Suppl. Fig. 2). Thus, purified CPS 3 is immunogenic in mice when adjuvanted with TiterMax Gold® and leads to an opsonizing IgG antibody response.

Purified CPS 3 immunogenicity is not influenced by phenol-extractable immune-stimulating ligands

As we showed that CPS 3 induces a type 1 IgG antibody response *in vivo*, we decided to investigate the presence of potential TLR ligands in the native CPS 3 preparation. As shown above, no native *S. suis* CPS, CPS 3 included, led to the production of IL-6 or TNF by CPS-stimulated DCs, in contrast to the cytokine production observed with *S. pneumoniae* CPS 19F (Fig. 3). In our study, the production of both IL-6 and TNF was dependent upon MyD88 signaling, but independent of TLR2, indicating that the CPS 19F preparation used contains TLR ligands (Suppl. Fig. 3). Following phenol extraction, *S. suis* CPS 3 did not show structural alterations by dot-blot and by ^{1}H NMR analyses (Suppl. Fig. 4 & 5). DCs stimulated with phenol-extracted CPS 3 secreted no IL-6 nor TNF in the supernatant, and their CCL2 and CCL3 production remained unchanged when compared to its native counterpart (data not shown).

To further determine if potential TLR ligands affected the antibody response *in vivo*, mice received on days 0 and 21 a dose of 25 µg of either native or phenol-extracted CPS 3 adjuvanted with TiterMax Gold®. Kinetic analysis of total Ig [IgG + IgM] antibody responses against native CPS 3 at days −1, 20, and 41 showed no significant differences ($P > 0.05$) between mice immunized with native and phenol-extracted CPS 3 (data not shown). Similarly, no significant differences were observed in anti-CPS 3 antibody isotypes (total Ig, IgM, and IgG) titers in sera collected on day 42 ($P > 0.05$; Fig. 7). These results suggest that immunogenicity of purified native CPS 3 *in vivo* is independent from phenol-extractable immune-stimulating ligands.

In vivo anti-CPS responses to heat-killed encapsulated intact S. suis serotypes 2, 3, and 9

Following the previous immunizations performed using soluble antigens, we also investigated the CPS-specific antibody response in the context of encapsulated intact bacteria. Titers of the different anti-CPS antibody isotypes were determined in mice that were hyperimmunized by multiple weekly intraperitoneal injections with 1×10^{9} CFU of heat-killed *S. suis*. Since mice hyperimmunized with *S. suis* serotypes 7 and 8 showed no anti-CPS responses (Suppl. Fig. 1D–E), these two serotypes were not further studied. Pooled serum

from mice hyperimmunized with *S. suis* serotype 2 showed intermediate levels of IgM and low levels of IgG that are specific to CPS 2, with barely detectable levels of IgG2b and low levels of IgG2c as the only IgG subclasses detected (Fig. 8A). In contrast, pooled serum from mice hyperimmunized with *S. suis* serotype 3 possessed high levels of IgM and intermediate levels of IgG specific to CPS 3 (Fig. 8B). Analysis of the anti-CPS 3 IgG subclasses showed high levels of IgG2c in contrast to barely detectable to low levels of IgG1, IgG2b, and IgG3. Finally, pooled serum from mice hyperimmunized with *S. suis* serotype 9 possessed intermediate levels of IgM and strong levels of IgG specific to CPS 9 (Fig. 8C). Analysis of the anti-CPS 9 IgG subclasses revealed high levels of IgG2b and IgG2c, in addition to low levels of IgG1 and IgG3. For all three serotypes, IgG2c was the dominant IgG subclass induced in hyperimmunized mice (Fig. 8).

In vivo anti-CPS responses to live encapsulated intact S. suis serotypes 2, 3, and 9

While titration of sera from mice hyperimmunized with heat-killed *S. suis* provided interesting data, we also studied the CPS-specific antibody response following infections with live *S. suis*, as this is the most relevant model. In the present study, a sublethal infection model was used to better characterize the antibody responses in the absence of significant inflammation and severe systemic clinical signs. During pretrials, different bacterial doses were tested, and sublethal doses producing little to no mortality and/or severe clinical signs of disease were chosen for each strain (data not shown). Titration of the different anti-CPS antibody isotypes after primary infection with *S. suis* serotype 3 revealed strong levels of IgM and low levels of IgG ($P \leq 0.01$) in comparison to placebo mice (Fig. 9A–C). A secondary infection with this serotype showed a significant boost for the IgM ($P \leq 0.05$) but not for the IgG ($P > 0.05$) response. In contrast to serotype 3, mice infected with *S. suis* serotype 2 showed no anti-CPS antibody titers following a primary infection, whereas a secondary infection induced low levels of IgM only ($P \leq 0.01$) (Fig. 9G–I). Sera from mice infected with *S. suis* serotype 9 displayed low levels of IgM and IgG that were not significant ($P > 0.05$) in comparison to placebo mice after a primary infection but nonetheless were boosted after a secondary infection ($P > 0.05$) (Fig. 9J–L). As such, whereas sublethal infections of mice with *S. suis* serotypes 2 and 9 induce low IgM anti-CPS responses only after a secondary infection, a sublethal infection with *S. suis* serotype 3 induce a high primary production of anti-CPS IgM

which was further boosted after a secondary infection. Anti-CPS IgG responses were only observed for serotypes 3 (as a primary response) and 9 (as a secondary response).

CPS 3 immunogenicity following sublethal infections is not influenced by the bacterial subcapsular domain

Since we demonstrated that CPS 3 is immunogenic by itself (as a soluble purified antigen) but also when associated with the bacterial body (as a particulate antigen), we also opted to evaluate if the immunogenicity of CPS 3 could be influenced by the composition and/or architecture of the bacterial subcapsular domain.

Titration of the different anti-CPS antibody isotypes in sera from mice that were sublethally infected with the 2to3 mutant revealed strong levels of IgM ($P \leq 0.001$) and no significant levels of IgG ($P > 0.05$) after a primary infection in comparison to placebo (Fig. 9D–F). After a secondary infection with the 2to3 mutant, a significant boost for the IgM ($P \leq 0.05$) and a significant IgG response ($P \leq 0.05$) were observed. Comparison of IgM and IgG responses in mice sublethally infected with either *S. suis* serotype 3 or the 2to3 mutant after primary and secondary infections showed no significant difference between the two strains ($P > 0.27$), although the primary IgG response was lower in mice infected with the 2to3 mutant (Fig. 9C & F). As such, the immunogenicity of CPS 3 associated with the whole bacteria appears to be independent from the bacterial subcapsular domain.

Discussion

The original idea behind this study stems from the fact that CPSs 2 and 14 of *S. suis* are particularly non-immunogenic, even when associated at the bacterial surface in the course of a primary or booster experimental infection (11, 25, 26, 47-49). This low immunogenicity results in undetectable (CPS 14) to low IgM (CPS 2) titers and undetectable IgG titers for both CPSs (25, 26). Also, this low immunogenicity cannot just be explained by the presence of sialic acid, known to possess immunomodulatory properties (24, 26, 50). Following recent reports by our team of new repeating unit structures for *S. suis* CPSs, especially serotypes 3, 7, 8, and 9, we decided to further investigate the potential immunogenicity of these serotypes, as

they are structurally well diversified among themselves and from the previously described sialylated serotypes.

Purified polysaccharides are known to be TI antigens and thus are generally poorly immunogenic. By inducing multivalent B cell receptor (mIg) cross-linking, TI antigens can induce proliferation of B cells and limited IgM responses lacking isotype switching. However, in the presence of a second signal such as TLR signaling, B cells activated by TI-antigens undergo isotype switching and secrete substantial amounts of Ig (14). A previous study by Sen *et al.* have demonstrated that pneumococcal polysaccharide preparations used for vaccine production contain TLR2 and TLR4 ligands, and that these TLR ligands substantially enhance primary and secondary anti-pneumococcal polysaccharide (PPS) responses in mice, especially the type 1 IgG subclasses (20). As such, those unappreciated co-purified and/or contaminating TLR ligands in PPS vaccine preparations play an important role in providing the second signal for the induction of an *in vivo* IgG TI humoral response to purified polysaccharides.

Despite the importance of CPS as protective antigens and on the development of antibody responses, few studies have been dedicated to the characterization of CPS activity on APCs and to the corresponding signaling mechanisms. And of those that are available, one must be cautious, as many of these studies describing innate immune activity of various purified polysaccharides have failed to rigorously rule out contaminating immune-stimulating ligands (14). It would be logical to assume that structural features of CPSs, such as different repeating unit compositions or glycosidic linkages, are susceptible to producing different immune responses, although such mechanisms remain largely unknown. Our results demonstrated that *S. suis* purified CPSs of serotypes 1, 1/2, 3, 7, 8, and 9 do not induce the release of key pro-inflammatory cytokines, which confirms their poor stimulatory activity. However, all these CPSs, including *S. pneumoniae* CPS 19F, stimulated DC production of two members of the CC family of chemokines, *i.e.* CCL2 and CCL3. These two chemokines are known to play a major role in the selective recruitment of monocytes, macrophages, DCs, and lymphocytes to sites of inflammation and also known to be produced in the course of *S. suis* infections (24, 51, 52). Following stimulation with purified CPSs of *S. suis* and *S. pneumoniae*, DC production of CCL2 was shown to be independent from TLR2 and MyD88

pathways, whereas CCL3 production was partially dependent upon both TLR2 and MyD88. These observations are in agreement with previous results obtained using purified CPSs of *S. suis* serotypes 2 and 14 and of GBS serotypes III and V (24), suggesting that DCs appear to respond to CPSs in a patterned manner, seemingly independent from their different structural features. This warrants further investigations into the involvement of members from the large family of lectin receptors as possible candidates (53-55).

Mouse immunizations demonstrated that purified CPS 3 when adjuvanted with TiterMax Gold®, a water-in-oil emulsion, induces the development of strong opsonizing type 1 IgG (mainly IgG2b and IgG3) and IgM antibody responses, in stark contrast to CPSs 2, 7, 8, 9 (this study), and 14 (26). Immunization of purified CPS 3 alone or combined with Alhydrogel® or with Quil-A® as adjuvants failed to mount an antibody response. Nonetheless, this makes CPS 3 as the first immunogenic purified CPS of *S. suis*. The current model for polysaccharide-specific TI humoral immunity requires two signals to ensure antibody secretion and class switching (14). Signal 1 is delivered through multivalent B cell receptor cross-linking by polymeric TI antigens. Signal 2 is delivered to CPS-specific B cells by innate cells, notably NK cells and γδ T cells, through cytokine (such as IFN-γ, GM-CSF, and IL-3) and/or BAFF/APRIL production, which are induced in response to PAMPs (such as TLR ligands). However, we showed that our purified CPS preparations are exempt from phenol-extractable contaminants and from nucleic acids and proteins, and also that they fail to induce cytokine production by DCs, at least those evaluated in our study. Another possible source for Signal 2 *in vivo* could be from the adjuvants used. A previous study has shown that stimulation of porcine DCs with TiterMax Gold® with no antigen induced IL-6 production, whereas stimulation with either Alhydrogel 2%® or Quil-A® induced IL-6 and/or IL-12 production, suggesting that these adjuvants by themselves can trigger Signal 2 following immunization with purified CPSs (56). Yet, only the combination of CPS 3 with TiterMax Gold® induced a potent protective response. This could be explained by the particulate form of the water-in-oil emulsion. Relative to soluble antigens, antigens in particulate form are phagocytosed more efficiently, produce higher levels of immune activation, and deliver concentrated antigens per particule than could be achieved through pinocytosis of soluble antigens (57, 58). Of note, aluminum salt-based adjuvants, such as Alhydrogel 2%®, are

themselves particulate, but antigens adsorbed to them do not behave as particulates (57, 58). Nevertheless, the adjuvant properties of TiterMax Gold® were not beneficial to the immunogenicity of other CPSs studied. Taken together, our results suggest that purified CPS 3 possesses unique structural and/or biochemical properties. In this regard, it was observed that water soluble CPS 3 was precipitated in the presence of the detergent sodium deoxycholate, suggesting an amphiphilic character for this polysaccharide (59). The hydrophobic element is most likely the di-*N*-acetylated D-bacillosamine, as acetylation increases hydrophobicity (such as is the case for chitin vs. chitosan (60)), which is then linked to the negatively charged D-glucuronic acid, together forming the amphiphilic disaccharide repeating unit of CPS 3 (Fig. 1). In turn, this amphiphile could be co-presented at the water-oil interface and/or associate with the amphiphilic block copolymer component of the TiterMax Gold® emulsion, in a similar fashion to its presentation at bacterial surfaces, and fully benefit from its adjuvant properties (61). Nonetheless, further studies into the structural and biochemical properties of purified CPS 3 and their effect on the induction of humoral immunity are warranted.

The immunogenicity of CPS 3 was also demonstrated in mice injected with serotype 3 intact whole bacteria, either heat-killed via hyperimmunization or live via sublethal infections. Mice infected/immunized with the serotype 3 bacteria produced a very robust IgM anti-CPS antibody response in addition to isotype-switched IgGs. Additionally, and in contrast to soluble purified CPS, a very strong anti-CPS 9 IgG response was observed in mice hyperimmunized with heat-killed serotype 9, whereas mice sublethally infected showed a weak IgM and IgG response only after a secondary dose. Due to the virulence of the serotype 9 strain used for sublethal infections, lower doses had to be employed, which might explain the lower response observed. This displayed immunogenicity for CPSs 3 and 9 in the context of whole intact bacteria is in agreement with the previously mentioned model for polysaccharide-specific TI humoral immunity: as CPSs are displayed at the bacterial surfaces, there are many bacterial PAMPs, such as TLR2 ligands, to trigger Signal 2 by APCs leading to stronger antibody secretion and isotype switching (14). Differences in these signals might also explain divergences in IgG switching, IgG subclasses, and/or presence/amplitude of memory (boost) anti-CPS responses between serotypes. Immunization/infection with intact serotype 2 whole bacteria led to the lowest anti-CPS response (in quantity and quality). Yet, a recipient

serotype 2 strain displaying a serotype 3 CPS induced optimal anti-CPS 3 antibody response, confirming the poorly immunogenic nature of CPS 2, and that anti-CPS 3 response to whole bacteria is independent from the bacterial subcapsular domain. A previous study has reported an important role of bacterial subcapsular domain in the anti-CPS response against *S. pneumoniae* serotype 14 by studying an asialo mutant of GBS serotype III, which expresses an identical CPS (62). The absence of this effect in our model could be due to the fact that intra-species serotype switching was used to better reflect serotype divergences within the *S. suis* bacterium.

Even though the predominant IgG subclasses differ between purified CPSs and intact bacteria for serotype 3, or among serotypes for hyperimmunized mice, the anti-CPS response remains biased towards the type 1 subclasses of IgG (IgG2a/IgG2c, IgG2b, and IgG3), which are known to be superior in both opsonophagocytosis activity and complement activation than the type 2 (IgG1) subclass (63, 64). Also, sublethal infections with serotypes 2, 3, and 9 led to boosted IgM and/or IgG titers following a secondary infection, a feature of T cell-dependent responses, in contrast to the TI response to purified CPS 3, which failed to show a significant boost following the second dose. Taken together, these observations demonstrate a number of consistent differences between intact bacteria in general *versus* purified CPSs, suggesting that different mechanisms are involved in the development of anti-CPS humoral responses (14, 62, 65-67).

The results obtained in this study using either purified CPSs or whole bacteria have strong implications for the design of effective vaccines against *S. suis* and provide novel insights into the complexity of the anti-CPS response. Our data further support the importance of the Th1 bias and/or the type 1 antibody response in the immuno-pathogenesis of *S. suis* infections and development of a protective response. Finally, this study provides important information on the divergent evolution of CPS serotypes with highly different structural and/or biochemical properties within *S. suis* and their interaction with the immune system.

Acknowledgements

The authors thank Ms. Évelyne Lacasse and Audrey Goyet for excellent technical assistance. The authors also wish to thank Dr. Masatoshi Okura (National Agriculture and Food Research Organization, Japan) for providing the CPS-switched 2to3 mutant of *S. suis* strain P1/7. This work was mainly supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a grant to MS (no. 342150). Partial support was provided by Canadian Glycomics Network, funded by Networks of Centres of Excellence of Canada (grant TP-6 to MS and MG). G. Goyette-Desjardins and J.-P. Auger are both recipients of a FRQNT Doctoral Scholarships and of an Alexander Graham Bell Graduate Scholarships—Doctoral Program from NSERC.

References

1. Gottschalk M, Xu J, Calzas C, Segura M. *Streptococcus suis*: a new emerging or an old neglected zoonotic pathogen? Future Microbiol. 2010;5(3):371-91.
2. Gottschalk M, Xu J, Lecours M-P, Grenier D, Fittipaldi N, Segura M. *Streptococcus suis* Infections in Humans: What is the prognosis for Western countries? (Part II). Clin Microbiol Newsl. 2010;32(13):97-102.
3. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect. 2014;3(6):e45.
4. Fittipaldi N, Segura M, Grenier D, Gottschalk M. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent *Streptococcus suis*. Future Microbiol. 2012;7(2):259-79.
5. Roy D, Auger JP, Segura M, Fittipaldi N, Takamatsu D, Okura M, et al. Role of the capsular polysaccharide as a virulence factor for *Streptococcus suis* serotype 14. Can J Vet Res. 2015;79(2):141-6.
6. Vilaichone RK, Vilaichone W, Nunthapisud P, Wilde H. *Streptococcus suis* infection in Thailand. J Med Assoc Thai. 2002;85:S109-17.
7. Kerdsin A, Hatrongjit R, Gottschalk M, Takeuchi D, Hamada S, Akeda Y, et al. Emergence of *Streptococcus suis* serotype 9 infection in humans. J Microbiol Immunol Infect. 2017;50(4):545-6.
8. Segura M. Fisher scientific award lecture - The capsular polysaccharides of Group B *Streptococcus* and *Streptococcus suis* differently modulate bacterial interactions with dendritic cells. Can J Microbiol. 2012;58(3):249-60.
9. Bottomley MJ, Serruto D, Sáfadi MAP, Klugman KP. Future challenges in the elimination of bacterial meningitis. Vaccine. 2012;30(Supplement 2):B78-B86.
10. Song JY, Moseley MA, Burton RL, Nahm MH. Pneumococcal vaccine and opsonic pneumococcal antibody. J Infect Chemother. 2013;19(3):412-25.

11. Goyette-Desjardins G, Calzas C, Shiao TC, Neubauer A, Kempker J, Roy R, et al. Protection against *Streptococcus suis* serotype 2 infection using a capsular polysaccharide glycoconjugate vaccine. Infect Immun. 2016;84(7):2059-75.
12. Takeuchi O, Akira S. Pattern Recognition Receptors and Inflammation. Cell. 2010;140(6):805-20.
13. Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. 1991;9:271-96.
14. Snapper CM. Differential regulation of polysaccharide-specific antibody responses to isolated polysaccharides, conjugate vaccines, and intact Gram-positive versus Gram-negative extracellular bacteria. Vaccine. 2016;34(30):3542-8.
15. d'Empaire G, Baer MT, Gibson FC. The K1 Serotype Capsular Polysaccharide of *Porphyromonas gingivalis* Elicits Chemokine Production from Murine Macrophages That Facilitates Cell Migration. Infect Immun. 2006;74(11):6236-43.
16. Wang Q, McLoughlin RM, Cobb BA, Charrel-Dennis M, Zaleski KJ, Golenbock D, et al. A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2. J Exp Med. 2006;203(13):2853-63.
17. Simpson SQ, Singh R, Bice DE. Heat-killed pneumococci and pneumococcal capsular polysaccharides stimulate tumor necrosis factor-alpha production by murine macrophages. Am J Respir Cell Mol Biol. 1994;10(3):284-9.
18. Soell M, Diab M, Haan-Archipoff G, Beretz A, Herbelin C, Poutrel B, et al. Capsular polysaccharide types 5 and 8 of Staphylococcus aureus bind specifically to human epithelial (KB) cells, endothelial cells, and monocytes and induce release of cytokines. Infect Immun. 1995;63(4):1380-6.
19. Zughaier SM. Neisseria meningitidis capsular polysaccharides induce inflammatory responses via TLR2 and TLR4-MD-2. J Leukoc Biol. 2011;89(3):469-80.
20. Sen G, Khan AQ, Chen Q, Snapper CM. In Vivo Humoral Immune Responses to Isolated Pneumococcal Polysaccharides Are Dependent on the Presence of Associated TLR Ligands. J Immunol. 2005;175(5):3084-91.
21. Yu J, Briles DE, Englund JA, Hollingshead SK, Glezen WP, Nahm MH. Immunogenic protein contaminants in pneumococcal vaccines. J Infect Dis. 2003;187(6):1019-23.
22. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. Biochem Cell Biol. 2010;88(3):513-25.
23. Van Calsteren MR, Gagnon F, Calzas C, Goyette-Desjardins G, Okura M, Takamatsu D, et al. Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide. Biochem Cell Biol. 2013;91(2):49-58.
24. Calzas C, Goyette-Desjardins G, Lemire P, Gagnon F, Lachance C, Van Calsteren MR, et al. Group B *Streptococcus* and *Streptococcus suis* capsular polysaccharides induce chemokine production by dendritic cells via Toll-like receptor 2- and MyD88-dependent and -independent pathways. Infect Immun. 2013;81(9):3106-18.
25. Calzas C, Lemire P, Auray G, Gerdts V, Gottschalk M, Segura M. Antibody response specific to the capsular polysaccharide is impaired in *Streptococcus suis* serotype 2-infected animals. Infect Immun. 2015;83(1):441-53.
26. Calzas C, Taillardet M, Fourati I, Roy D, Gottschalk M, Soudeyns H, et al. Evaluation of the immunomodulatory properties of *Streptococcus suis* and Group B *Streptococcus* capsular polysaccharides on the humoral response. Pathogens. 2017;6(2):16.

27. Van Calsteren MR, Goyette-Desjardins G, Gagnon F, Okura M, Takamatsu D, Roy R, et al. Explaining the serological characteristics of *Streptococcus suis* serotypes 1 and 1/2 from their capsular polysaccharide structure and biosynthesis. J Biol Chem. 2016;291(16):8387-98.
28. Vinogradov E, Goyette-Desjardins G, Okura M, Takamatsu D, Gottschalk M, Segura M. Structure determination of *Streptococcus suis* serotype 9 capsular polysaccharide and assignment of functions of the *cps* locus genes involved in its biosynthesis. Carbohydr Res. 2016;433:25-30.
29. Goyette-Desjardins G, Vinogradov E, Okura M, Takamatsu D, Gottschalk M, Segura M. *Streptococcus suis* serotype 3 and serotype 18 capsular polysaccharides contain di-N-acetyl-bacillosamine. Carbohydr Res. 2018;466:18-29.
30. Goyette-Desjardins G, Vinogradov E, Okura M, Takamatsu D, Gottschalk M, Segura M. Structure determination of *Streptococcus suis* serotypes 7 and 8 capsular polysaccharides and assignment of functions of the *cps* locus genes involved in their biosynthesis. Carbohydr Res. 2019;473:36-45.
31. Athey TB, Teatero S, Lacouture S, Takamatsu D, Gottschalk M, Fittipaldi N. Determining *Streptococcus suis* serotype from short-read whole-genome sequencing data. BMC Microbiol. 2016;16(1):162.
32. Roy D, Athey TBT, Auger J-P, Goyette-Desjardins G, Van Calsteren M-R, Takamatsu D, et al. A single amino acid polymorphism in the glycosyltransferase CpsK defines four *Streptococcus suis* serotypes. Sci Rep. 2017;7(1):4066.
33. Gottschalk M, Kolberg J, Charland N, Jacques M. *Streptococcus pneumoniae* types 19A and 19F and *Streptococcus suis* capsular type 8 share common capsular epitopes. J Clin Microbiol. 1995;33(9):2492-5.
34. Daniels CC, Rogers PD, Shelton CM. A Review of Pneumococcal Vaccines: Current Polysaccharide Vaccine Recommendations and Future Protein Antigens. The journal of pediatric pharmacology and therapeutics : JPPT : the official journal of PPAG. 2016;21(1):27-35.
35. Segura M, Stankova J, Gottschalk M. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect Immun. 1999;67(9):4646-54.
36. Segura M, Su Z, Piccirillo C, Stevenson MM. Impairment of dendritic cell function by excretory-secretory products: a potential mechanism for nematode-induced immunosuppression. Eur J Immunol. 2007;37(7):1887-904.
37. Lecours M-P, Gottschalk M, Houde M, Lemire P, Fittipaldi N, Segura M. Critical Role for *Streptococcus suis* Cell Wall Modifications and Suilysin in Resistance to Complement-Dependent Killing by Dendritic Cells. J Infect Dis. 2011;204(6):919-29.
38. Wernette CM, Frasch CE, Madore D, Carlone G, Goldblatt D, Plikaytis B, et al. Enzyme-Linked Immunosorbent Assay for Quantitation of Human Antibodies to Pneumococcal Polysaccharides. Clin Diagn Lab Immunol. 2003;10(4):514-9.
39. Goyette-Desjardins G, Roy R, Segura M. Murine whole-blood opsonophagocytosis assay to evaluate protection by antibodies raised against encapsulated extracellular bacteria. In: Lepenies B, editor. Carbohydrate-Based Vaccines - Methods and Protocols. Methods in Molecular Biology. 1331. New York, USA: Springer; 2015. p. 81-92.
40. Higgins R, Gottschalk M. An update on *Streptococcus suis* identification. J Vet Diagn Invest. 1990;2(3):249-52.

41. Wishart DS, Bigam CG, Yao J, Abildgaard F, Dyson HJ, Oldfield E, et al. 1H, 13C and 15N chemical shift referencing in biomolecular NMR. J Biomol NMR. 1995;6(2):135-40.
42. Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol. 2010;17(7):1055-65.
43. Baker CJ, Paoletti LC, Wessels MR, Guttormsen H-K, Rench MA, Hickman ME, et al. Safety and Immunogenicity of Capsular Polysaccharide—Tetanus Toxoid Conjugate Vaccines for Group B Streptococcal Types Ia and Ib. J Infect Dis. 1999;179(1):142-50.
44. Pawlowski A, Kallenius G, Svenson SB. Preparation of pneumococcal capsular polysaccharide-protein conjugate vaccines utilizing new fragmentation and conjugation technologies. Vaccine. 2000;18(18):1873-85.
45. Li Y, Gottschalk M, Esgleas M, Lacouture S, Dubreuil JD, Willson P, et al. Immunization with Recombinant Sao Protein Confers Protection against Streptococcus suis Infection. Clin Vaccine Immunol. 2007;14(8):937-43.
46. Esgleas M, Dominguez-Punaro MC, Li Y, Harel J, Dubreuil JD, Gottschalk M. Immunization with SsEno fails to protect mice against challenge with *Streptococcus suis* serotype 2. FEMS Microbiol Lett. 2009;294(1):82-8.
47. Elliott SD, Clifton-Hadley F, Tai J. Streptococcal infection in young pigs. V. An immunogenic polysaccharide from *Streptococcus suis* type 2 with particular reference to vaccination against streptococcal meningitis in pigs. J Hyg (Lond). 1980;85(2):275-85.
48. del Campo Sepulveda EM, Altman E, Kobisch M, D'Allaire S, Gottschalk M. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect ELISA. Vet Microbiol. 1996;52(1-2):113-25.
49. Wisselink HJ, Stockhofe-Zurwieden N, Hilgers LAT, Smith HE. Assessment of protective efficacy of live and killed vaccines based on a non-encapsulated mutant of *Streptococcus suis* serotype 2. Vet Microbiol. 2002;84(1–2):155-68.
50. Varki A, Gagneux P. Multifarious roles of sialic acids in immunity. Ann N Y Acad Sci. 2012;1253(1):16-36.
51. Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol. 2004;36(10):1882-6.
52. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009;29(6):313-26.
53. Mayer S, Raulf M-K, Lepenies B. C-type lectins: their network and roles in pathogen recognition and immunity. Histochem Cell Biol. 2017;147(2):223-37.
54. Varki A, Angata T. Siglecs—the major subfamily of I-type lectins. Glycobiology. 2006;16(1):1R-27R.
55. Thiemann S, Baum LG. Galectins and Immune Responses-Just How Do They Do Those Things They Do? Annu Rev Immunol. 2016;34:243-64.
56. Martelet L, Lacouture S, Goyette-Desjardins G, Beauchamp G, Surprenant C, Gottschalk M, et al. Porcine dendritic cells as an in vitro model to assess the immunological behaviour of *Streptococcus suis* subunit vaccine formulations and the polarizing effect of adjuvants. Pathogens. 2017;6(1).
57. Snapper CM. Distinct Immunologic Properties of Soluble Versus Particulate Antigens. Front Immunol. 2018;9:598.
58. Colino J, Chattopadhyay G, Sen G, Chen Q, Lees A, Canaday DH, et al. Parameters Underlying Distinct T Cell-Dependent Polysaccharide-Specific IgG Responses to an

Intact Gram-Positive Bacterium versus a Soluble Conjugate Vaccine. J Immunol. 2009;183(3):1551-9.
59. Kuttel MM, Cescutti P, Distefano M, Rizzo R. Fluorescence and NMR spectroscopy together with molecular simulations reveal amphiphilic characteristics of a Burkholderia biofilm exopolysaccharide. J Biol Chem. 2017;292(26):11034-42.
60. Ravi Kumar MNV. A review of chitin and chitosan applications. Reactive and Functional Polymers. 2000;46(1):1-27.
61. Newman MJ, Balusubramanian M, Todd CW. Development of adjuvant-active nonionic block copolymers. Adv Drug Deliv Rev. 1998;32(3):199-223.
62. Arjunaraja S, Paoletti LC, Snapper CM. Structurally Identical Capsular Polysaccharide Expressed by Intact Group B Streptococcus versus Streptococcus pneumoniae Elicits Distinct Murine Polysaccharide-Specific IgG Responses In Vivo. J Immunol. 2012.
63. Michaelsen TE, Kolberg J, Aase A, Herstad TK, Høiby EA. The four mouse IgG isotypes differ extensively in bactericidal and opsonophagocytic activity when reacting with the P1.16 epitope on the outer membrane PorA protein of *Neisseria meningitidis*. Scand J Immunol. 2004;59(1):34-9.
64. Collins AM. IgG subclass co-expression brings harmony to the quartet model of murine IgG function. Immunol Cell Biol. 2016;94(10):949-54.
65. Arjunaraja S, Massari P, Wetzler LM, Lees A, Colino J, Snapper CM. The Nature of an In Vivo Anti-Capsular Polysaccharide Response Is Markedly Influenced by the Composition and/or Architecture of the Bacterial Subcapsular Domain. J Immunol. 2012;188(2):569-77.
66. Snapper CM. Differential Regulation of Protein- and Polysaccharide-Specific Ig Isotype Production In Vivo in Response to Intact Streptococcus pneumoniae. Current Protein & Peptide Science. 2006;7(4):295-305.
67. Snapper CM. Mechanisms underlying in vivo polysaccharide-specific immunoglobulin responses to intact extracellular bacteria. Ann N Y Acad Sci. 2012;1253(1):92-101.
68. Perch B, Pedersen KB, Henrichsen J. Serology of capsulated streptococci pathogenic for pigs: six new serotypes of *Streptococcus suis*. J Clin Microbiol. 1983;17(6):993-6.
69. Slater JD, Allen AG, May JP, Bolitho S, Lindsay H, Maskell DJ. Mutagenesis of *Streptococcus equi* and *Streptococcus suis* by transposon Tn917. Vet Microbiol. 2003;93(3):197-206.
70. Chuzeville S, Auger JP, Dumesnil A, Roy D, Lacouture S, Fittipaldi N, et al. Serotype-specific role of antigen I/II in the initial steps of the pathogenesis of the infection caused by *Streptococcus suis*. Vet Res. 2017;48(1):39.
71. Varki A, Cummings RD, Aebi M, Packer NH, Seeberger PH, Esko JD, et al. Symbol Nomenclature for Graphical Representations of Glycans. Glycobiology. 2015;25(12):1323-4.
72. Jennings HJ, Rosell K-G, Carlo DJ. Structural determination of the capsular polysaccharide of *Streptococcus pneumoniae* type 19 (19F). Can J Chem. 1980;58(11):1069-74.

Tables

Table 1. Bacterial strains of *Streptococcus suis* used in this study.

Strain	Serotype	General characteristics	Used for	References
S735 (ATCC 43765)	2	Reference strain for serotype 2 CPS production. Strain isolated from a clinical case of swine infection in Denmark	CPS purification Mouse hyperimmunization	(22, 81)
P1/7 (ATCC BAA-853)	2	WT. Well-encapsulated serotype 2 strain isolated from a clinical case of swine infection in the United Kingdom	Mouse sublethal infections	(82)
2to3 Mutant	3	CPS-switched mutant derived from P1/7 strain. The *cps*2 locus was replaced by *cps*3 locus.	Mouse sublethal infections	Dr. M. Okura, unpublished data
1178027	1	Reference strain for serotype 1 CPS production. Strain isolated from a clinical case of swine infection in Canada	CPS purification	(27)
2651	1/2	Reference strain for serotype 1/2 CPS production. Strain isolated from a clinical case of swine infection in Denmark	CPS purification	(27)
4961	3	Reference strain for serotype 3 CPS production. Strain isolated from a clinical case of swine infection in Denmark	CPS purification Mouse hyperimmunization Opsonophagocytosis assay	(29)

			Mouse sublethal infections	
1750775	7	Reference strain for serotype 7 CPS production. Strain isolated from a clinical case of swine infection in Canada	CPS purification Mouse hyperimmunization Coating of ELISA plates with *S. suis*	(30)
1719887	8	Reference strain for serotype 8 CPS production. Strain isolated from a clinical case of swine infection in Canada	CPS purification Mouse hyperimmunization Coating of ELISA plates with *S. suis*	(30)
1273590	9	Reference strain for serotype 9 CPS production. Strain isolated from a clinical case of swine infection in Canada	CPS purification Mouse hyperimmunization Coating of ELISA plates with *S. suis*	(28)
1135776	9	Well-encapsulated serotype 9 strain isolated from a clinical case of swine infection in Canada	Mouse sublethal infections	(83)

Table 2. Quality control tests of purified *S. suis* and *S. pneumoniae* CPSs.

CPS	% (w/w) nucleic acids[1]	% (w/w) proteins	
		Modified Lowry Assay[2]	NanoOrange Assay[3]
S. suis serotype 2	0.4	< 0.5	< 0.4
S. suis serotype 1	0.4	0.6	ND
S. suis serotype 1/2	0.2	< 0.5	ND
S. suis serotype 3 native	0.4	0.5	< 0.4
S. suis serotype 3 phenol-extracted	0.2	< 0.5	< 0.4
S. suis serotype 7	0.2	< 0.5	ND
S. suis serotype 8	0.2	< 0.5	ND
S. suis serotype 9	0.5	6.7	< 0.4
S. pneumoniae serotype 19F	0.2	< 0.5	ND

[1] Determined by spectrophotometry at 230 and 260 nm.

[2] Determined by the Modified Lowry Protein Assay Kit. Values that were below the limit of detection are shown as < 0.5% (w/w).

[3] Determined by the NanoOrange Protein Quantitation Kit. Values that were below the limit of detection are shown as < 0.4% (w/w). ND, not determined.

Figures

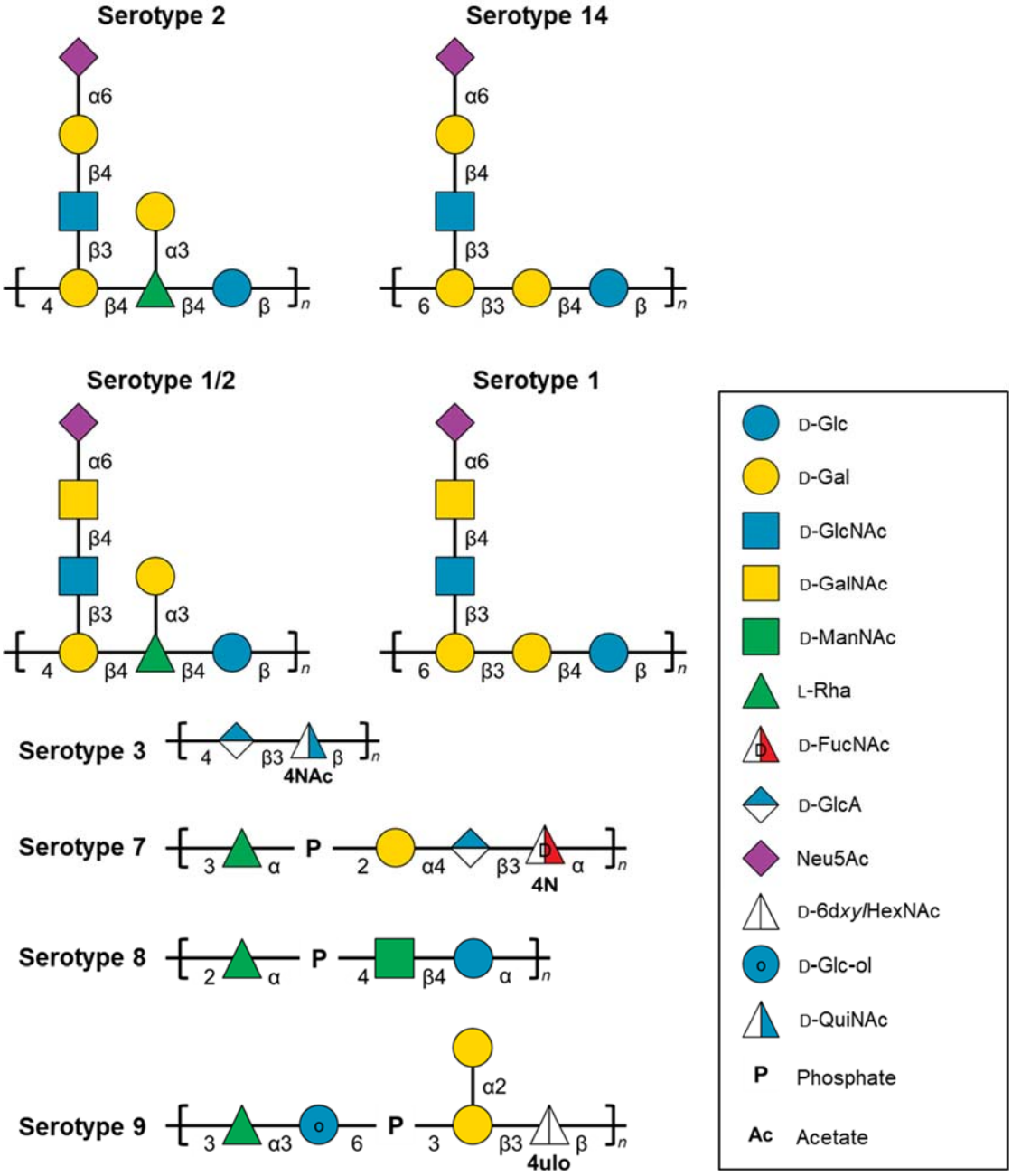


Figure 1. Repeating unit structures of *S. suis* capsular polysaccharides that were included in this study: serotypes 2 (22), 14 (23) 1/2 & 1 (27), 3 (29), 7 & 8 (30), and 9 (28). Monosaccharide symbols follow the Symbol Nomenclature for Glycans (SNFG) system (79). Abbreviation: 2-acetamido-2,6-dideoxy-β-D-*xylo*-hexopyranose (D-6d*xyl*HexNAc).

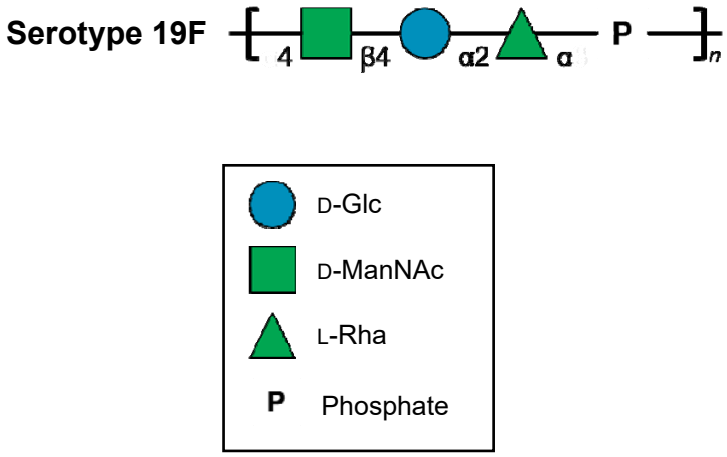


Figure 2. Repeating unit structure of *S. pneumoniae* serotype 19F capsular polysaccharide that was included in this study (80).

Monosaccharide symbols follow the SNFG system (79).

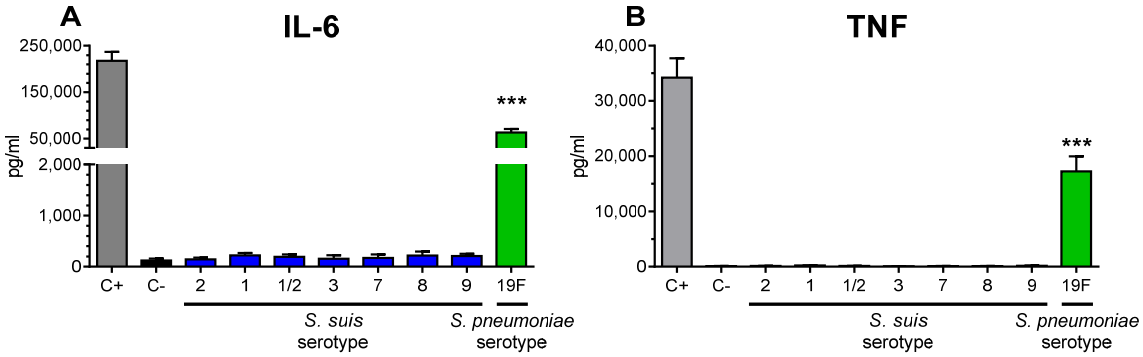


Figure 3. Pro-inflammatory cytokine production by DCs in response to stimulation by *S. suis* or *S. pneumoniae* CPSs for 24 h.

Native CPSs of *S. suis* serotypes 2, 1, 1/2, 3, 7, 8, and 9 or of *S. pneumoniae* serotype 19F (each at 200 µg/ml) were incubated with DCs (10^6 cells/ml). After 24 h, supernatants were collected, and IL-6 (A) and TNF (B) levels were quantified by ELISA. Cells stimulated with medium alone and with LPS (1 µg/ml) served as negative (C-) and positive (C+) controls, respectively. Data are expressed as means ± SEM for at least three experiments. Statistically significant differences *versus* medium alone (C-) are indicated as follows: ***, $P \leq 0.001$.

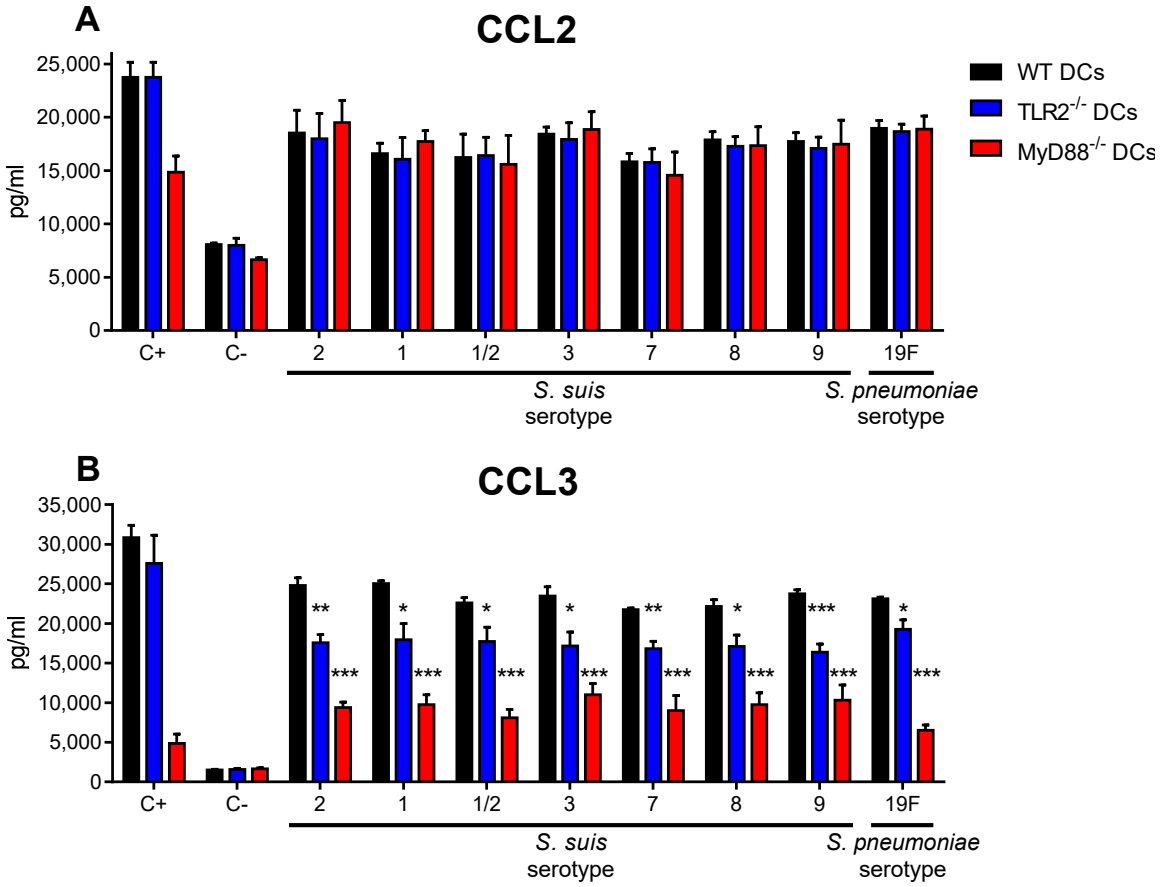


Figure 4. Role of TLR2 and of MyD88 in chemokine production by DCs in response to stimulation by *S. suis* or *S. pneumoniae* CPSs for 24 h.

Native CPSs of *S. suis* serotypes 2, 1, 1/2, 3, 7, 8, and 9 or of *S. pneumoniae* serotype 19F (each at 200 µg/ml) were incubated with either WT (black bars), TLR2$^{-/-}$ (blue bars) or MyD88$^{-/-}$ (red bars) DCs (10^6 cells/ml). After 24 h, supernatants were collected, and CCL2 (A) and CCL3 (B) levels were quantified by ELISA. Cells stimulated with medium alone and with LPS (1 µg/ml) served as negative (C-) and positive (C+) controls, respectively. Data are expressed as means ± SEM for at least three experiments. Statistically significant differences *versus* the WT are indicated as follows: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

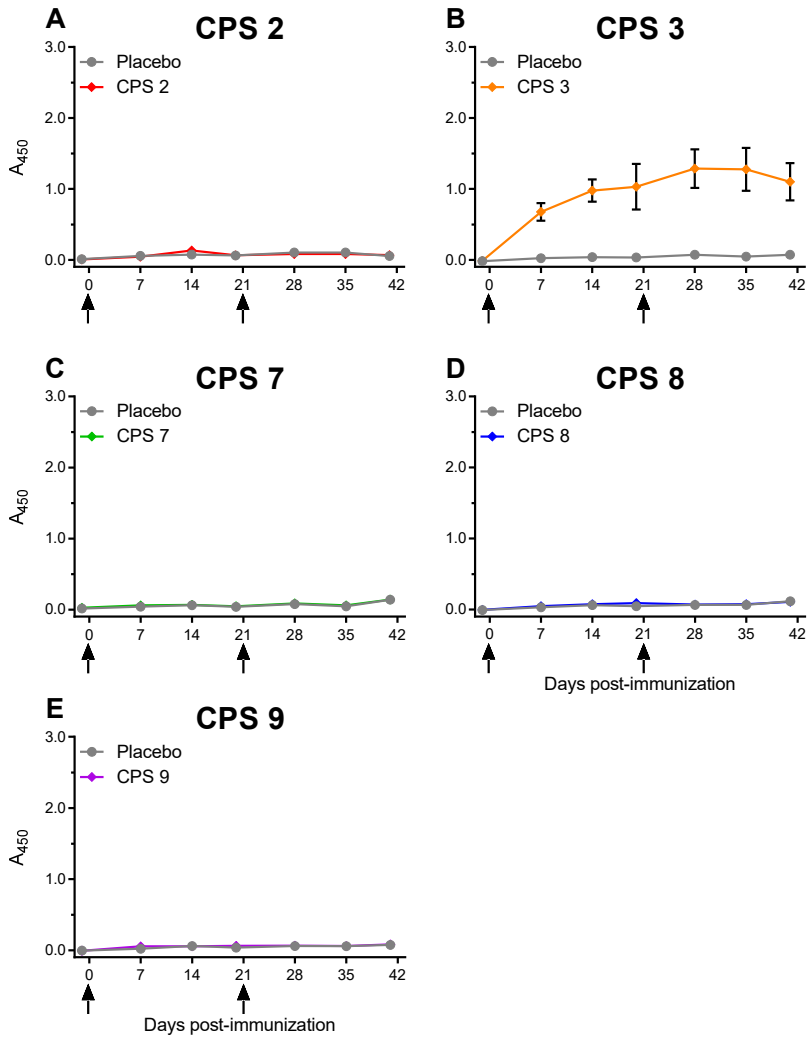


Figure 5. Kinetics of total antibody responses of mice immunized with 25 µg of either *S. suis* serotype 2 (A), serotype 3 (B), serotype 7 (C), serotype 8 (D), or serotype 9 (E) native purified CPS adjuvanted with TiterMax Gold®.

Mice ($N = 10$) were immunized on day 0 and boosted on day 21. Placebo mice ($N = 10$) were similarly injected with PBS adjuvanted with TiterMax Gold®. ELISA plates were coated with native CPS from its respective serotype and incubated with blood samples diluted 1:100 to measure anti-CPS antibodies. Total Ig [IgG + IgM] antibody levels are shown as means ± SEM of absorbance values at 450 nm. Placebo groups are shown by a grey circle, whereas the CPS-immunized groups are shown by a colored diamond. The arrow at day 21 indicates the boost.

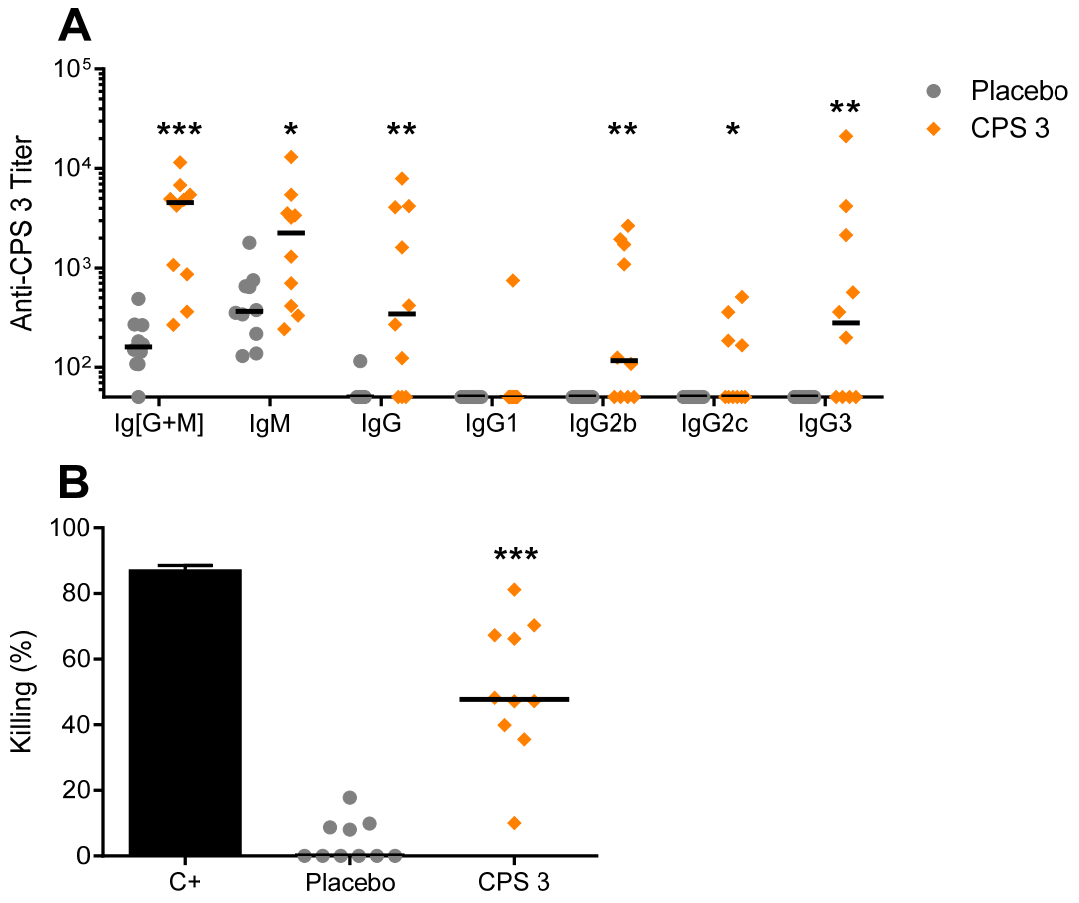


Figure 6. Isotyping and functional studies of antibodies produced in mice immunized with native *S. suis* serotype 3 purified capsular polysaccharide (CPS 3) in TiterMax Gold®.

Mice (N = 10) were immunized on day 0 and boosted on day 21 with 25 µg of CPS 3. Placebo mice (N = 10) were similarly injected with PBS adjuvanted with TiterMax Gold®. Sera were collected on day 42. (A) For titration of anti-CPS antibody isotypes, ELISA plates were coated with native CPS 3 and incubated with 2-fold serial dilutions of sera, and isotypes were detected using specific HRP-conjugated anti-mouse total Ig [IgG + IgM], IgM, IgG, IgG1, IgG2b, IgG2c, or IgG3 antibodies. Results are expressed as titer for individual mice, with horizontal bars representing median. (B) Opsonophagocytosis killing of *S. suis* type 3 strain 4961 by day 42 sera from mice immunized with 25 ug of CPS 3 adjuvanted with TiterMax Gold®. A 40% (v/v) sample serum and a bacterial MOI of 0.1 were added to fresh whole

blood from naive mice to perform the assay. Viable bacterial counts were determined after 2 h of incubation. To determine bacterial killing, viable bacterial counts from tubes incubated with sample sera were compared to those incubated with control naive mouse sera. Rabbit anti-*S. suis* type 3 strain 4961 serum was used as a positive control (C+) and compared to control naive rabbit serum to determine bacterial killing. Results are expressed as percent bacterial killing for individual mice, with horizontal bars representing median. Individuals from the placebo group are shown by a grey circle, whereas those of the CPS 3 group are shown by an orange diamond. Statistically significant differences *versus* the placebo group are indicated as follows: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

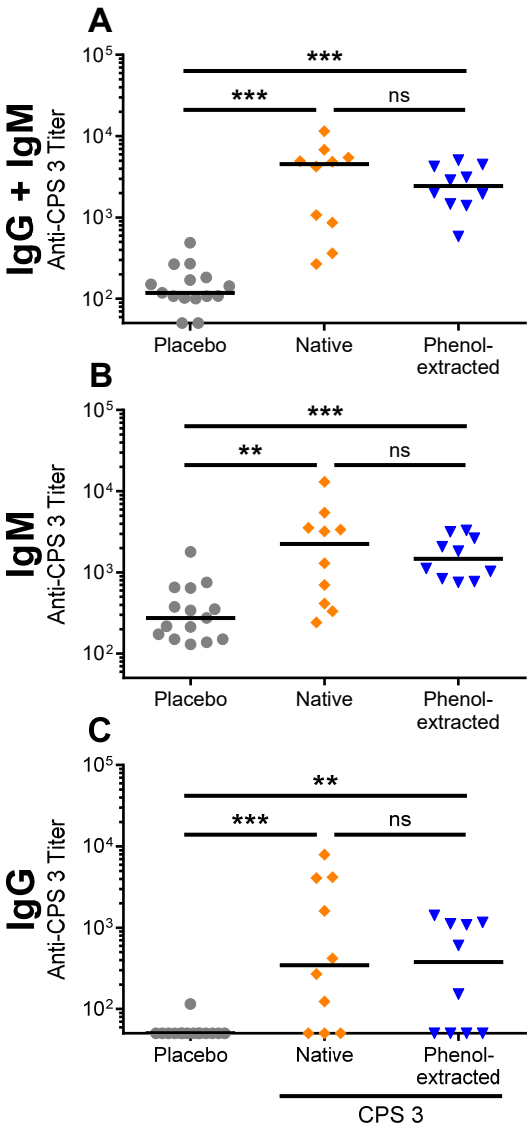


Figure 7. Isotyping of antibodies produced in mice immunized with phenol-extracted *S. suis* serotype 3 purified capsular polysaccharide (CPS 3) in TiterMax Gold®.

Mice ($N = 10$) were immunized on day 0 and boosted on day 21 with 25 µg of either native or phenol-extracted CPS 3. Placebo mice ($N = 15$) were similarly injected with PBS adjuvanted with TiterMax Gold®. Sera were collected on day 42. For titration of anti-CPS antibody isotypes, ELISA plates were coated with native CPS 3 and incubated with 2-fold serial dilutions of sera, and isotypes were detected using specific HRP-conjugated anti-mouse total Ig [IgG + IgM], IgM, or IgG antibodies. Results are expressed as titer for individual mice,

with horizontal bars representing median. Individuals from the placebo group are shown by a grey circle, those of the native CPS 3 group are shown by an orange diamond, and those of the phenol-extracted group are shown by a blue triangle. Statistically significant differences between groups are indicated as follows: ns, $P > 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

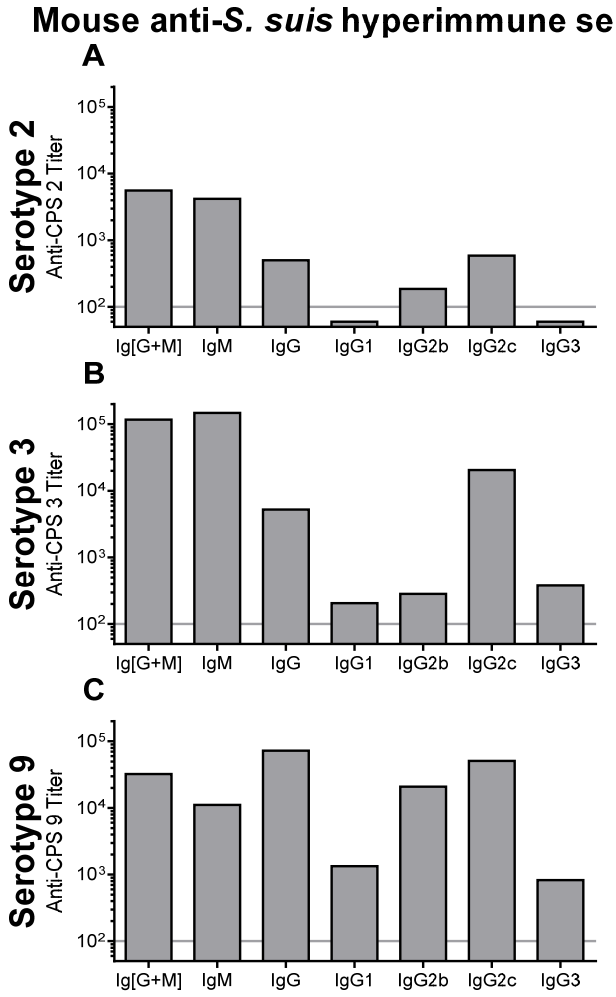


Figure 8. Isotyping of antibodies induced in control mice hyperimmunized with heat-killed *S. suis* serotype 2 strain S735 (A), serotype 3 strain 4961 (B), and serotype 9 strain 1273590 (C).

Hyperimmune mice (N = 3–4) were obtained by repeated immunization with 1×10^9 CFU/ml heat-killed *S. suis* in THB by intraperitoneal injection weekly during 4 to 8 weeks. Two weeks after the last injection, sera were collected and pooled by serotype. For titration of anti-CPS antibody isotypes, ELISA plates were coated with native CPS 2, CPS 3, or CPS 9 and incubated with 2-fold serial dilutions of sera, and isotypes were detected using specific HRP-conjugated anti-mouse total Ig [IgG + IgM], IgM, IgG, IgG1, IgG2b, IgG2c, or IgG3 antibodies. Results are expressed as titer, and the horizontal line behind the bars represents the cutoff; values found below are considered negative (titer < 100).

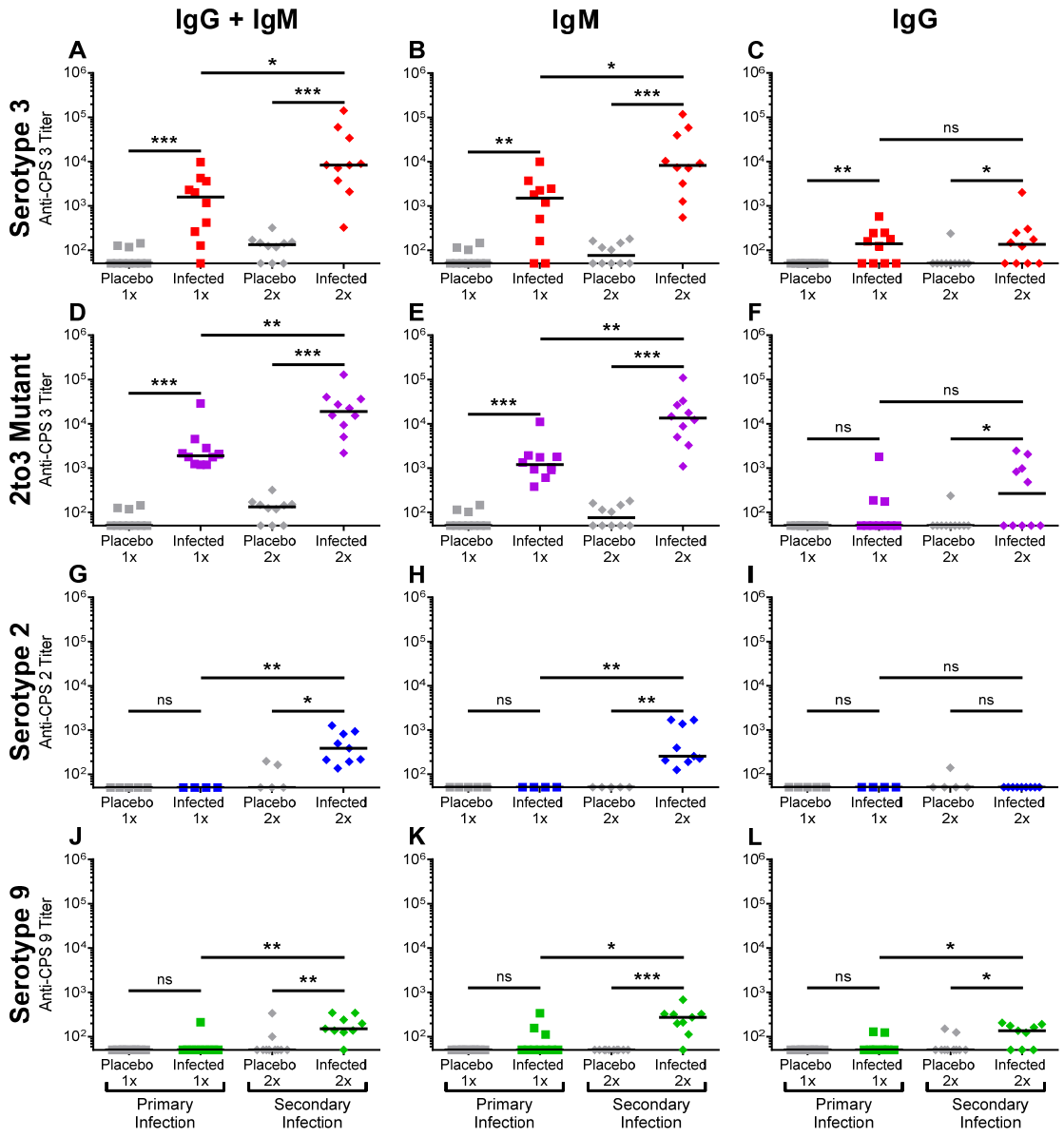


Figure 9. Anti-CPS antibody response after infections of mice with sublethal doses of *S. suis* serotype 3 strain 4961 (1 × 10^6 CFU; A–C), a CPS-switched from serotype 2to3 mutant of strain P1/7 (1 × 10^6 CFU; D–F), serotype 2 strain P1/7 (1 × 10^6 CFU; G–I), and serotype 9 strain 1135776 (1 × 10^5 CFU; J–L).

For the primary infection (1x), mice were infected on day 0 followed by sera collection on day 21. For the secondary infection (2x), mice were infected on day 0, reinfected on day 21, then sera was collected on day 42. Placebo mice were similarly injected once or twice with the vehicle solution (sterile Todd-Hewitt broth). Mouse groups were as follows: mice infected 1x

or 2x with the serotype 3 and 2to3 mutant and corresponding placebo mice (N = 10); mice infected with the serotype 2 (1x, N = 4; 2x, N = 9) and corresponding placebo mice (N = 5); mice infected with the serotype 9 (1x, N = 10; 2x, N = 9) and corresponding placebo mice (N = 10). For titration of anti-CPS antibody isotypes, ELISA plates were coated with serotype-matched native CPS and incubated with 2-fold serial dilutions of sera, and isotypes were detected using specific HRP-conjugated anti-mouse total Ig [IgG + IgM], IgM, IgG antibodies. Results are expressed as titer for individual mice, with horizontal bars representing median. Individuals from the placebo groups are shown by a grey symbol, whereas those of the infected groups are shown by a colored symbol. Statistically significant differences between groups are indicated as follows: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

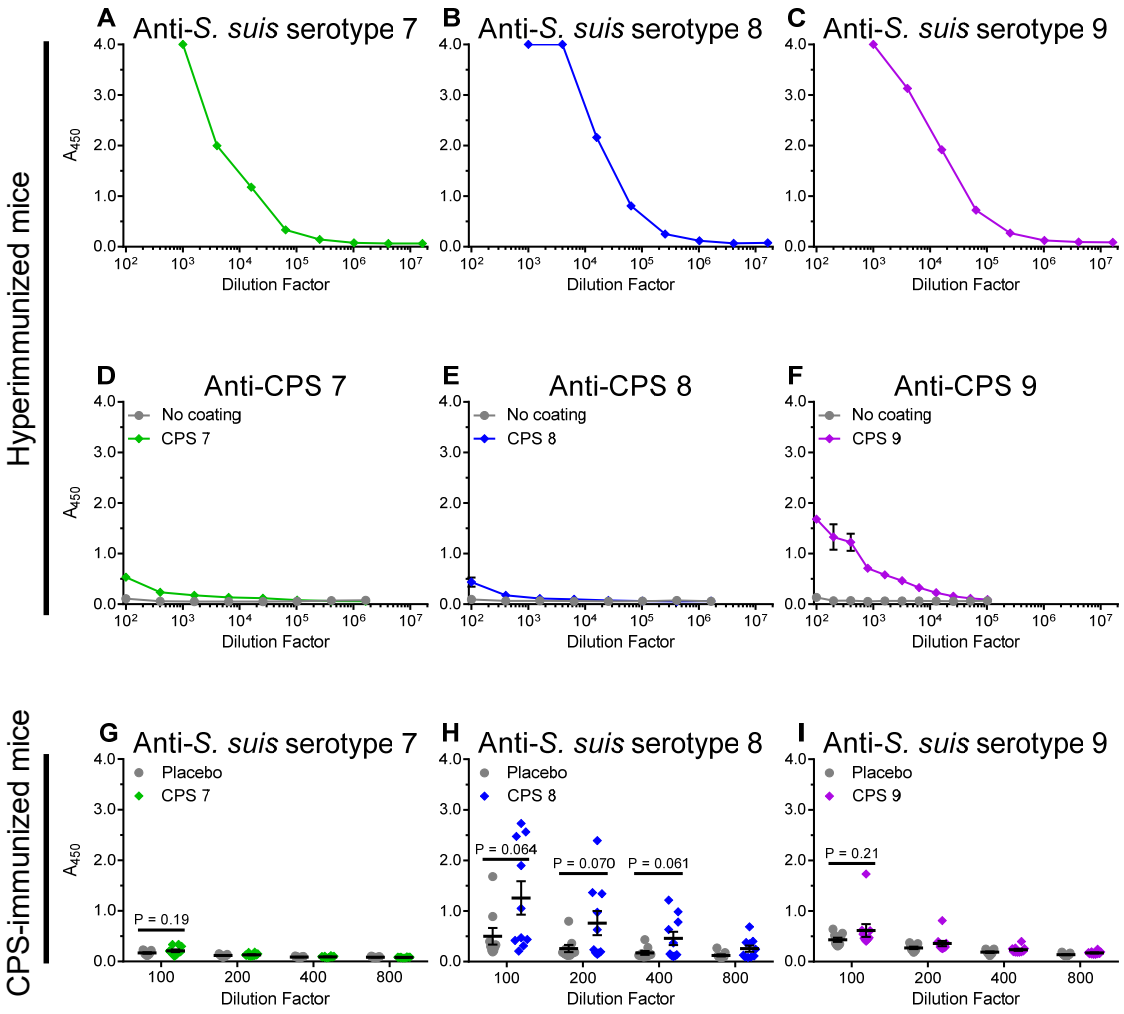


Figure S1. Additional controls for the total antibody response of mice immunized with purified CPSs of *S. suis* serotype 7 (CPS 7), serotype 8 (CPS 8), and serotype 9 (CPS 9) (see Fig. 5).

(A–C) Total anti-*S. suis* response of control sera from hyperimmunized mice. Hyperimmune mice (N = 3–4) were obtained by repeated immunization with 1 × 10^9 CFU/ml heat-killed *S. suis* in THB by intraperitoneal injection weekly during 4 to 8 weeks. Two weeks after the last injection, sera were collected and pooled by serotype. ELISA plates were coated with whole bacteria of either *S. suis* serotype 7 strain 1750775 (A), serotype 8 strain 1719887 (B), or serotype 9 strain 1273590 (C) and incubated with serial dilutions of sera from hyperimmunized mice. Antibodies were detected using specific HRP-conjugated anti-mouse

total Ig [IgG + IgM]. Results are shown as means ± SEM of absorbance values at 450 nm for three technical replicates. (D–F) Total anti-CPS response of control sera from hyperimmunized mice. For titration of total anti-CPS antibodies, ELISA plates were not coated or coated with native CPS 7 (D), CPS 8 (E), or CPS 9 (F) and incubated with serial dilutions of sera, and antibodies were detected using specific HRP-conjugated anti-mouse total Ig [IgG + IgM]. Results are shown as means ± SEM of absorbance values at 450 nm for three technical replicates. Responses from wells that were not coated are shown by a grey circle, whereas the CPS-coated wells are shown by a colored diamond. (G–I) Total anti-*S. suis* response of sera from mice immunized with 25 μg of either CPS 7 (G), CPS 8 (H), or CPS 9 (I) native purified CPS adjuvanted with TiterMax Gold®. Mice (N = 10) were immunized on day 0 and boosted on day 21. Placebo mice (N = 10) were similarly injected with PBS adjuvanted with TiterMax Gold®. Sera were collected on day 42. ELISA plates were coated with whole bacteria of either *S. suis* serotype 7 strain 1750775 (G), serotype 8 strain 1719887 (H), or serotype 9 strain 1273590 (I) and incubated with sera samples diluted 1:100, 1:200, 1:400, or 1:800. Antibodies were detected using specific HRP-conjugated anti-mouse total Ig [IgG + IgM]. Results are shown as absorbance values at 450 nm for individual mice, with horizontal bars representing means ± SEM. Individuals from the placebo groups are shown by a grey circle, whereas those of the immunized groups are shown by a colored diamond.

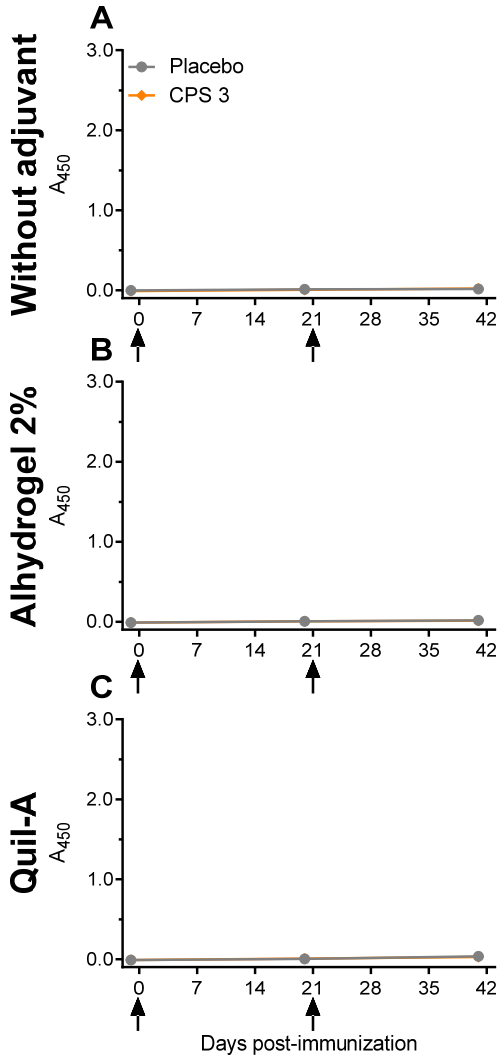


Figure S2. Kinetics of total antibody responses of mice immunized with 25 µg of native *S. suis* serotype 3 purified capsular polysaccharide (CPS 3) either non-adjuvanted or adjuvanted with Alhydrogel 2%® or Quil-A®.

Mice ($N = 10$) were immunized on day 0 and boosted on day 21. Placebo mice ($N = 10$) were similarly injected with PBS only or PBS adjuvanted with Alhydrogel 2%® or Quil-A®. ELISA plates were coated with native CPS 3 and incubated with blood samples diluted 1:100 to measure anti-CPS antibodies. Total Ig [IgG + IgM] antibody levels are shown as means ± SEM of absorbance values at 450 nm. Placebo groups are shown by a grey circle, whereas the CPS 3-immunized groups are shown by an orange diamond. The arrow at day 21 indicates the boost.

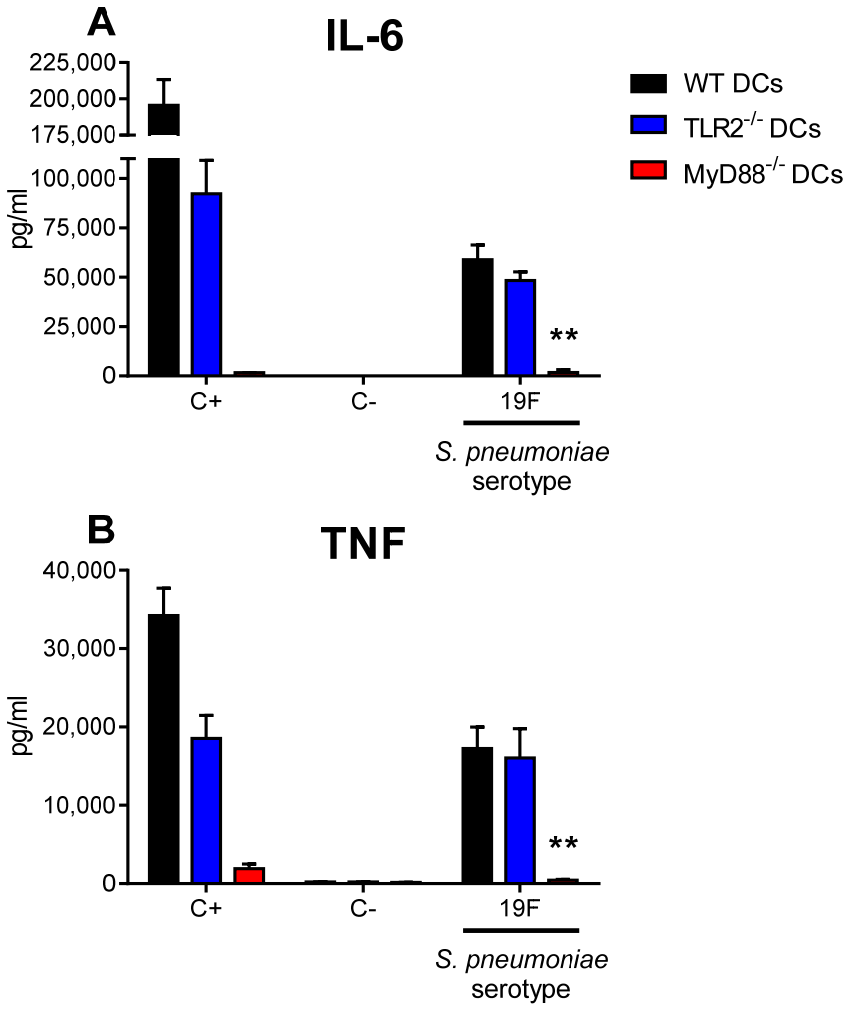


Figure S3. Role of TLR2 and of MyD88 in cytokine production by DCs in response to stimulation by *Streptococcus pneumoniae* CPS for 24 h.

CPS of *S. pneumoniae* serotype 19F (at 200 µg/ml) was incubated with either WT (black bars), TLR2$^{-/-}$ (blue bars) or MyD88$^{-/-}$ (red bars) DCs (10^6 cells/ml). After 24 h, supernatants were collected, and IL-6 (A) and TNF (B) levels were quantified by ELISA. Cells stimulated with medium alone and with LPS (1 µg/ml) served as negative (C-) and positive (C+) controls, respectively. Data are expressed as means ± SEM for at least three experiments. Statistically significant differences *versus* the WT are indicated as follows: **, $P \leq 0.01$.

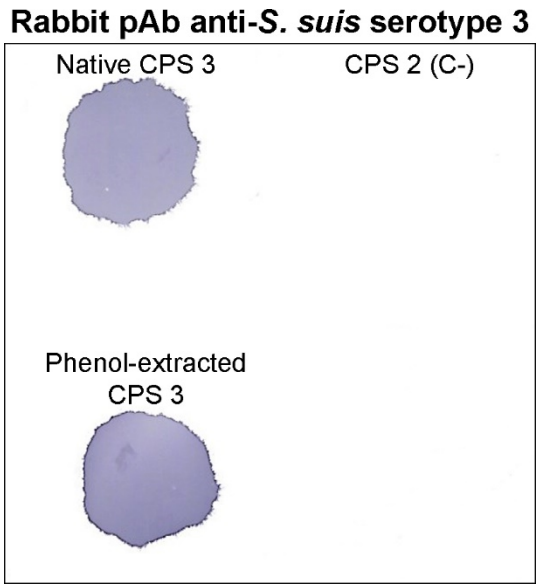


Figure S4. Dot-blot analysis with 10 µg of native and phenol-extracted capsular polysaccharides from *Streptococcus suis* serotype 3 (CPS 3) using rabbit anti-*S. suis* serotype 3 serum (pAb).

Purified CPS from *S. suis* serotype 2 (CPS 2) was used as a negative control (C-).

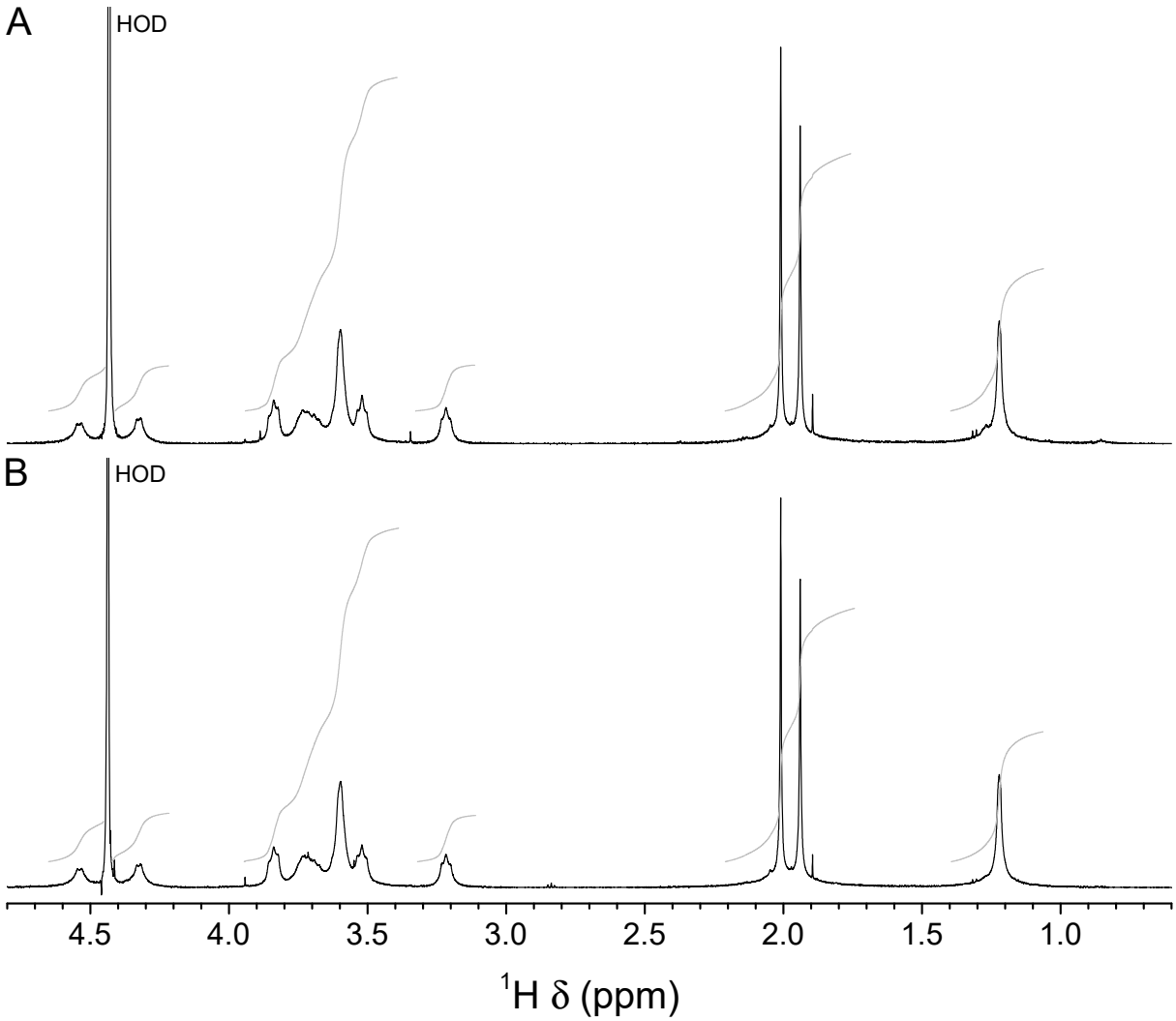


Figure S5. 500-MHz ^{1}H NMR spectra of *Streptococcus suis* serotype 3 native and phenol-extracted capsular polysaccharides (CPS 3) in D_2O at 60°C.

(A) Native CPS 3. (B) Phenol-extracted CPS 3.

ARTICLE V

Murine Whole-blood Opsonophagocytosis Assay to Evaluate Protection by Antibodies Raised Against Encapsulated Extracellular Bacteria

---

Guillaume Goyette-Desjardins [1], René Roy [2], and Mariela Segura [1]

[1] Laboratory of Immunology, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, Quebec, Canada, J2S 2M2;

[2] Canada Research Chair in Therapeutic Chemistry, Department of Chemistry, Université du Québec à Montréal, Montreal, Quebec, Canada, H3C 3P8.

Publié dans : Lepenies B, ed. *Carbohydrate-Based Vaccines - Methods and Protocols. Methods in Molecular Biology.* 1331. New York, USA: Springer; **2015**. p. 81-92.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de ce chapitre de livre. J'ai participé activement à la conception du modèle, à la standardisation du protocole (100%) et à l'écriture du chapitre.

IMPORTANCE DE CETTE CONTRIBUTION

Les tests d'opsonophagocytoses sont employés pour évaluer la protection d'un individu suite à une immunisation pour prévenir les infections contre différentes bactéries, notamment *S. pneumoniae*, GBS et *N. meningitidis*. Ceux-ci permettent d'évaluer la fonctionnalité des anticorps induits et sont préférés aux titres déterminés par ELISA. Les essais déjà décrits emploient normalement des lignées cellulaires ou des types cellulaires purifiés, ce qui sous-estime la complexité de l'activité bactéricide du sang. Ici, nous avons développé un test d'opsonophagocytose employant du sang entier de souris afin de pallier ces limites. Comme la plupart des techniques rapportées dans les articles de recherche sont souvent trop peu détaillées pour être reproduites convenablement, la principale force de cette contribution est que celle-ci a été publiée sous forme de chapitre de livre, permettant d'incluant tous les détails pertinents pour être reproduite par d'autres.

Ainsi, la versatilité de ce nouveau test d'opsonophagocytose a pu être démontrée dans le cadre de cette thèse, notamment pour évaluer la fonctionnalité d'anticorps murins dirigés contre le sérotype 2 de *S. suis* suite à l'immunisation par un vaccin glycoconjugué (**ARTICLES VI et VII**). De plus, celui-ci a été employé pour étudier d'autres sérotypes de *S. suis*, comme le sérotype 3 (**ARTICLE IV**) et les sérotypes 1, 1/2, 9 et 14 (**ARTICLE VII**). De plus, en adaptant ce test pour *S. pneumoniae* (sérotypes 8 et 14), nous avons entretenu une fructueuse collaboration avec l'équipe du Dr Mario Feldman ayant menée à la publication de deux articles dans des journaux à facteur d'impact élevé (**Annexes, ARTICLES XVII et XVIII**). D'autant plus, la méthode décrite a également été adaptée avec succès pour le sang entier de porc au sein de notre laboratoire, permettant d'évaluer la fonctionnalité d'anticorps porcins dirigés contre le sérotype 7 de *S. suis* après vaccination par une bactérine au sein d'une ferme de maternité.

Ainsi, le test d'opsonophagocytose rapporté ici (**ARTICLE V**) est hautement versatile et facilement être adapté pour l'étude de différents pathogènes bactériens et de différentes espèces animales. De plus, celui-ci a permis l'obtention de plusieurs résultats rapportés dans cette thèse, tel que détaillé ci-haut.

Summary

In vaccine development, especially against pathogenic encapsulated extracellular bacteria, functional assays such the opsonophagocytosis assay (OPA) are preferred to ELISA titers for evaluating protection against infection. Such assays are normally performed using phagocytic cell lines or purified cell types, which underestimates the complexity of blood bactericidal activity. Here, we describe an OPA using murine whole-blood as effector cells, in a small format (0.2 ml), which requires small quantities of sera (80 µl or less) from immunized individuals. Easy to develop and perform, this OPA can be readily adapted to various pathogens and could be used to evaluate sera from human or animal clinical trials.

1. Introduction

In vaccine development, correlates of immunity correspond to measurable signs showing that an individual is protected against an infection, such as specific antibody titers or as functional antibody activity (1-3). The use of an opsonophagocytosis assay (OPA) as correlate of immunity is preferred to ELISA titers for evaluating protection against invasive bacterial diseases, such as pneumonia, meningitis and septicemia (4). OPAs have been mainly described for several encapsulated Gram-positive bacteria, including *Streptococcus pneumoniae*. OPA is based on the fact that opsonization by specific immunoglobulins (antibodies) at the bacterial surface will activate the classical pathway of complement, leading to complement deposition (5). Together, deposited immunoglobulins and/or complement components will be recognized by Fc receptors and complement receptors, respectively, triggering an enhanced immune response by blood leukocytes which results in bacterial phagocytosis and bactericidal activity (6-8). Specific cell type activation depends on the immunoglobulin isotypes present in the immune serum, since each isotype possesses different binding preferences to Fc receptors, which differently influences the cell response (8). As such, OPAs are performed by mixing and incubating effector cells (phagocytes), the target (bacteria) and specific antibodies (naive or immune sera). The presence of specific and functionally active antibodies in the test sera will result in target elimination by effector cells. For Gram-negative bacteria, the generally accepted format is the serum bactericidal assay, which uses serum complement as the sole effector for bacterial killing (9-11), mostly due to the important role played by the complement membrane attack complex in the direct lysis of Gram-negative bacteria (12). However, OPA, as correlate of immunity, have also been applied to some Gram-negative encapsulated bacteria, such as *Neisseria meningitidis* (13).

While most OPAs are performed using phagocytic cell lines, here we describe a method for such a test using a more complete murine whole-blood model (4). Instead of using a cell line or a purified single cell type, the OPA requires whole blood from naive mice. This model takes into account all blood leukocytes present and thus represents a more realistic model of the complex interactions between all immune cells, plasmatic proteins/components, and bacteria during a systemic infection. Other advantages of this assay are the use of small

volumes of reagents (only 80 µl of diluted whole-blood and 80 µl or less of serum for a final volume of 200 µl) and the fact that the same assay microtube can be used for multiple time-points.

To develop this method, we used *Streptococcus pneumoniae* serotype 14 and *Streptococcus suis* serotype 2 as target bacterial models. *Streptococcus pneumoniae* serotype 14 is one of the most important serotypes causing invasive pneumococcal disease and is still included in all multivalent vaccines (14). *Streptococcus suis* serotype 2 is an important swine pathogen and zoonotic agent, being the most frequently isolated and associated with disease, for which no vaccine is currently available (15). Both pathogens are extracellular, and antibodies against surface-exposed bacterial components (such as capsular polysaccharides and cell wall proteins) play a major role in host defense to fight these infections (5-8). We also present results for the negative and positive controls used for the OPA with *S. pneumoniae* serotype 14 and *S. suis* serotype 2. This developed method can be applied to other extracellular bacterial species, especially when measuring the activity of antibodies generated by glycoconjugate vaccines and directed against bacterial surface carbohydrates.

2. Materials

Prepare all solutions using fresh ultrapure deionized water (such as milli-Q purified) and analytical grade reagents. All materials and reagents that are to be in contact with the bacteria used in the assay must be sterile and endotoxin-free (*see* **Note 1**). All manipulations, except before sterilization, must be performed aseptically either using a flame or under a biological cabinet. Prepare or store all reagents at 4°C (unless indicated otherwise).

2.1. Bacterial growth, preparation and viable counts

1. Columbia Agar with 5% sheep blood (Oxoid, Nepean, ON, Canada).
2. Todd-Hewitt Broth (THB; Becton Dickinson, Mississauga, ON, Canada). Prepare according to the manufacturer's instructions. Autoclave to sterilize.
3. Sterile 15 ml polypropylene conical tubes.
4. Sterile 100 mm polystyrene Petri Dishes.

5. Todd-Hewitt Broth Agar (THA) plates. Dissolve 30 g of THB (Becton Dickinson) in 1000 ml of water. Add 15 g of agarose. Autoclave to sterilize and dissolve agar. Keep the solution warm in a water bath heated to 65°C until plates are poured. Pour 15 ml of THA per plate aseptically. Let the plates cool down for about one hour until solidified, then incubate for 24 h at 37°C without CO_2 to confirm the absence of plate contamination. Store plates in their plastic sleeves at 4°C.
6. Sterile and endotoxin/pyrogen-free phosphate buffered saline (PBS): 0.01 M $H_2PO_4^-$ /HPO_4^{2-}, 0.138 M NaCl, 0.0027 M KCl, pH 7.4 (Gibco/Invitrogen, Burlington, ON, Canada).
7. Sterile 1.5 ml microtubes (Eppendorf, Hamburg, Germany).
8. 1.5 ml microtubes containing either 180 µl or 900 µl of PBS for serial dilutions. Store at room temperature.

2.2. Blood collection and OPA

1. Sodium heparin solution: 140 USP/ml sodium heparin in PBS. Consult the lot certification for the specific activity (in USP/mg). Weight the required quantity and dissolve in 5 ml of sterile PBS. Aseptically filter through a 0.22 µm syringe filter and aliquot 50 µl of the sterile sodium heparin solution into 1.5 ml Eppendorf microtubes.
2. Five to eight week-old female C57BL/6 mice (*see* **Note 2**).
3. Sterile 1 ml syringes mounted with a sterile 25 G 5/8'' needle.
4. 5 ml polypropylene conical tubes (Eppendorf) (*see* **Note 3**).
5. 1.5 ml polypropylene microtubes (Eppendorf).
6. Complete RPMI medium: RPMI 1640 (no glutamine, no HEPES, with phenol red) supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine and 50 µM 2-mercaptoethanol (Gibco/Invitrogen). To 500 ml of RPMI 1640, add 25 ml of heat-inactivated fetal bovine serum (*see* **Note 4**), 5 ml of 1 M HEPES, 5 ml of 200 mM L-glutamine and 0.45 ml of 55 mM 2-mercaptoethanol.
7. Red Cell Lysis Buffer (eBiosciences, San Diego, California, USA).
8. Trypan blue solution.
9. Hemacytometer.
10. Inverted microscope for cell counting.

11. Sterile 25 G 5/8'' needle.

3. Methods

3.1. Bacterial culture and preparation for OPA

Here we present a general protocol for the growth and preparation of *Streptococci*. Please adapt growth medium and optimal growth conditions to your pathogen accordingly. All microbiological manipulations should be performed aseptically using either a flame or under a biological cabinet (*see* **Note 1**). Follow diligently all governmental and institutional regulations regarding the manipulation of pathogens.

1. On day -3, prepare a fresh bacterial culture by plating on blood agar. Incubate for 16-24 h at 37°C with 5% CO_2 (*see* **Note 5**).
2. On day -2, check the purity of the culture. Plate 3 colonies using an inoculation loop onto a new blood agar plate and incubate for 16-24 h at 37°C with 5% CO_2.
3. On day -1, check the purity of the culture. Inoculate 5 ml of THB using an inoculation loop with 3 colonies. Mix the contents of the tube for a few seconds using a vortex. Incubate the tubes standing for 16 h at 37°C with 5% CO_2.
4. On the day of the OPA, inoculate 10 ml of fresh THB with 0.1 ml from the previous 16 h-culture. Mix the tube for a few seconds using a vortex. Incubate the tubes standing at 37°C with 5% CO_2 until the culture reaches the mid-logarithmic phase.
5. Wash the bacteria by centrifuging at 9,000 x *g* for 5 min at 4°C and resuspending the pellet in 10 ml of PBS.
6. Repeat the wash a second time and resuspend the final pellet in 5 ml of PBS. Using a spectrophotometer, adjust the bacterial suspension by diluting with PBS in order to reach an OD_{600} = 0.6. Bacterial cultures must have been previously standardized in order to accurately determine the bacterial concentration in colony-forming units (CFU)/ml at this specific turbity. Once the culture conditions are standardized, always use the same protocol.
7. Perform serial dilutions in complete RPMI medium to obtain the desired bacterial concentration (in CFU/ml) for the assay (see **Table 1**). Keep the final suspension of

bacteria in complete RPMI medium on ice until you are ready to perform the OPA (*see* **Notes 6 and 7**).

[Table 1 near here]

3.2. Blood collection and preparation for OPA

All experiments and manipulations involving animals must be conducted in accordance with the governmental and institutional guidelines and policies. Personnel must be qualified to handle laboratory animals. All manipulations should be performed aseptically and all blood suspensions must be kept at room temperature (*see* **Note 8**).

1. Humanely euthanize one mouse at a time and collect blood by intracardiac puncture using a 1 ml syringe mounted with a 25 G 5/8'' needle (*see* **Note 9**).
2. Remove the needle and distribute the blood by adding approximately 450 µl of blood in the microtubes containing the sodium heparin solution (*see* **Note 10**).
3. Quickly mix the microtubes by gentle hand-agitation, 10 times (*see* **Note 11**).
4. Pool the blood recovered from all of the mice by transferring the blood from the microtubes to a 5 ml tube (*see* **Note 3**).
5. Count the leukocytes (white blood cells) from the pooled blood and dilute the pooled blood with complete RPMI medium to a final concentration of 6.25 x 10^6 leukocytes/ml (*see* **Note 12**).

3.3. Opsonophagocytosis assay (OPA)

All microbiological manipulations should be performed aseptically using either a flame or under a biological cabinet. Follow diligently all governmental and institutional regulations regarding the manipulation of pathogens and biological samples.

Internal controls: to perform the OPA, it is important to have, in advance, a positive control sera or polyclonal or monoclonal antibodies directed against the target pathogen. In addition, respective matching negative control sera or polyclonal or monoclonal antibodies must be included as negative control. The positive and negative control sera/antibodies will be used as internal controls (*see* **Note 13**). The concentration of positive and negative

control sera/antibodies to be added to the OPA must be standardized in advance. The goal is to obtain > 90 % of killing with the positive internal control.

The test sera must include not only the sera from placebo and immunized animal groups but also sera from naive (strict control) animals from the same animal lot as that used for the vaccine trial (*see* **Note 14**).

1. Distribute the diluted whole-blood (effector cells) and test sera (including naive, placebo and immunized sera) to the 1.5 ml microtubes. The volumes to be added to obtain the different OPA conditions are given in **Table 2**. Also include microtubes containing the standardized concentration of internal control sera/antibodies. Mix well the blood and sera together by gently tapping with a finger on the side of the microtube (*see* **Note 15**).
   [Table 2 near here]
2. Distribute the final suspension of bacteria to the assay microtubes. The concentrations for the 40 µl volumes to be added are given in **Table 1**. This step corresponds to the start of the assay (t = 0 min). Mix well the contents by gently tapping with a finger on the side of the microtube (*see* **Note 15**).
3. Pierce the top of every assay microtube with a sterile 25 G 5/8'' needle to allow cell respiration.
4. Incubate the assay microtubes at 37°C with 5% CO_2. Every 20 minutes, mix the microtube contents by gently tapping the side in order to increase contact between cells and bacteria (*see* **Note 15**). Optimal incubation times vary from one target pathogen to another and must be standardized.

3.4. Bacterial viable counts

All microbiological manipulations should be performed aseptically under a biological cabinet.

1. If several incubation times are being tested, you can use the same OPA microtube by retrieving a small volume at each time point. To this aim, at the desired incubation

time points, mix well each assay tube (by gently tapping on the side), retrieve 20 µl of the content and return the assay tube to the incubator. Dilute the 20 µl sample in a microtube containing 180 µl of PBS (10^{-1} dilution).

2. Mix the 10^{-1} dilution thoroughly by vortexing. Perform ten-fold serial dilutions by retrieving 100 µl from the previous dilution and diluting it in a microtube containing 900 µl of PBS. Mix thoroughly each microtube by vortexing (*see* **Note 16**).
3. Spread aseptically either 50 or 100 µl from selected dilutions on THA plates (*see* **Note 16**).
4. Incubate all plates for 24 h to 48 h at 37°C with 5% CO_2.
5. After incubation, count the CFU and determine the bacterial killing using the following formula:

$$\text{Killing \%} = \left(1 - \frac{\text{CFU from test serum}}{\text{CFU from naive serum}}\right)$$

Where "CFU from test serum" represents the CFU counts from one OPA test tube incubated with either placebo or immunized sera, and "CFU from naive serum" represents the average CFU counts from three OPA test tubes incubated with the pool of naive sera included each time the assay is performed (*see* **Note 17**).

The killing percentage represents the proportion of bacteria killed in a test tube compared to the tubes incubated with naive sera. In the case where CFU from test serum is higher than CFU from naive serum (resulting in a negative killing value), report killing percentage value as 0. The test sera (placebo and immunized) can be performed in duplicate or triplicate if the amount of serum sample is sufficient.

For internal controls, killing percentages are calculated using the same mathematical formula, except by using paired/matched positive and negative serum/antibodies as "test serum" and "naive serum" values, respectively.

3.5. Examples of results and further applications

Examples of results obtained with various positive and negative serum controls for *S. pneumoniae* and *S. suis* are shown in **Fig. 1** and **Fig. 2**, respectively. These results represent the different bacterial behaviors that would be observed during initial

standardization of the OPA and allow selection of optimal multiplicity of infection (MOI), incubation time and/or serum concentrations to be used.

The OPA method described in this chapter has been developed for mouse test sera and rabbit sera (mainly used as internal control). Immunoglobulins share common structures due to their function in immunity, thus enabling cross-species functional antibodies. As such, this OPA method could be easily adapted for other test sera, such as those from human vaccine clinical trials.

Notes

1. Working in sterile and endotoxin-free conditions will ensure intra- and inter-assay consistency. Microbial or endotoxin contamination might activate leukocytes and lead to high variations in the results.
2. Using mice with the same genetic background will ensure inter-assays consistency. Inbred mice, such as C57BL/6 or BALB/c, are recommended. Avoid using aged mice as the functionality of immune cells might be compromised. Both, male or female mice can be used. We preferred female mice as they are less aggressive than males for housing and handling.
3. A conical 15 ml tube is too long and too narrow in order to retrieve the diluted blood with a 1000 µl micropipette without risking contamination. Conical 5 ml tubes (Eppendorf) were found to work perfectly for this purpose.
4. To obtain the greatest reproducibility, when preparing cell culture medium always use certified serum grade, which is guaranteed to be exempt of endotoxin contamination,.
5. When starting a culture from a frozen aliquot, bacterial growth is more uniform and consistent after a minimum of 2 passages on blood agar. Blood agar plates used to start broth cultures can be kept up to one week at 4°C with parafilm to prevent dehydration. After one week of storage, a new blood agar plate should be inoculated.
6. When preparing the inoculum by diluting bacteria in complete RPMI medium, prepare at least an extra 0.5 ml for viable counts and purity verification.

7. If one person alone performs the assay, it is advisable to prepare, wash and dilute in complete RPMI medium the bacterial culture first, and keep it on ice while collecting mouse blood. During that waiting time, bacterial growth and/or death is greatly reduced if kept on ice. Nevertheless, this waiting time should not be longer than 3 to 4 hours. When the OPA assay is started, viable counts for the initial inoculum (at t = 0 min) can be performed. Control the purity of your final suspension by inoculating on blood agar.
8. Keeping blood suspensions at room temperature, instead of keeping on ice or at 4°C, helps to prevent hemolysis/cell lysis.
9. Depending on the age of the mice and the experience of the person perfoming the intracardiac puncture, 0.4 to 0.8 ml of blood is normally obtained from a single mouse. In this case, depending on number of tubes required for the assay, several mice are necessary in order to provide sufficient blood. It is recommended to perform one mouse at a time to maximize the amount of blood recovered per mouse.
10. It is important to remove the needle before flushing the blood out of the syringe to prevent lysis of effector cells. Indeed, flushing the blood through a narrow needle will cause animal cells to lyse.
11. From the moment the mouse is sacrificed, blood clotting begins. Thus, blood collection by intracardiac puncture, transfer to a microtube and mixing with the heparin solution must be performed as quickly as possible, in less than 3 minutes per mouse.
12. To count the leukocytes, aliquot 25 µl of the pooled blood in a microtube, add 250 µl of 1x Red Cell Lysis Buffer and incubate for 5 min at room temperature. After incubation, add 500 µl of room temperature PBS and quick spin at 13,000 x *g* for 12 sec at room temperature. Discard the supernatant, resuspend the cells with 25 µl of PBS, and dilute 1/20 with PBS followed by a 1/5 dilution with Trypan Blue (final dilution of 1/100). Determine the leukocyte concentration by counting the cells using a hemacytometer. We normally obtain approximately 7.5 x 10^6 leukocytes/ml of whole-blood.
13. Internal controls: when designing the assay, always include matching positive and negative sera, polyclonal antibodies or isotype-matched monoclonal antibodies as internal controls to ensure inter-assay consistency. These control sera/antibodies can

be produced in your own laboratory or purchased when commercially available for your pathogen. The species origin of these sera/antibodies can be different from mouse. For example, rabbit sera works very well as an internal control. If inconsistencies are observed in the values for positive and negative internal controls between tests, results should be discarded and the assay repeated. If inconsistencies persist, perform troubleshooting to find the problem (i.e. bacterial culture, cell viability, mouse lot, etc.)

14. Naive mouse sera: when testing immunized/placebo mouse sera from the vaccine trial, sera from a number of naive mouse of the same age, sex and genetic background (same lot if possible) might be collected and pooled to create the reference negative (naive) control used in the mathematical formula to calculate the % of killing. Do not use placebo sera as "naive sera" since animals might react to the injection with vehicle solution used as placebo (especially when containing adjuvants). Consequently, sera from placebo animals might give variable % of killing in the OPA test.
15. Whenever whole-blood is involved, mixing by using a vortex is highly discouraged. Such agitation will cause lysis of the leukocytes and result in a lower killing result.
16. To perform viable bacterial counts, long vortex mixing times such as ≥30 seconds are recommended to ensure maximal recovery of viable bacteria (internalized or not). In our experience, there was no need for an additional cell lysis step.The optimal dilutions to plate will depend on the chosen plating method (i.e. manual plating or automatic plating). It is recommended to use a large number of dilutions during preliminary standardization and then, based on these results, to select ranges of dilutions that will allow an accurate CFU counting. In the case of both negative and positive internal controls, a range of two dilutions will be sufficient. For the test sera, select at least four dilutions: two high range dilutions if a negative/low killing value is expected and two low range dilutions if a high % of killing is expected.
17. Naive sera assay tubes must be performed in each assay in triplicate in order to eliminate inter-tube variations. The average for the viable counts is then used in the formula to determine killing percentages.

Acknowledgements

This work was mainly supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a grant to MS (#342150). Partial contribution was also provided by Canadian Institutes of Health Research grant to MS and RR. The authors wish to thank Dr. Mario Feldman (University of Alberta) and Dr. Marcelo Gottschalk (University of Montreal) for their generous gifts of reference antisera and antibodies against *S. pneumoniae* and *S. suis*, respectively.

References

1. Plotkin SA. Immunologic correlates of protection induced by vaccination. Pediatr Infect Dis J. 2001;20(1):63-75.
2. Plotkin SA. Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis. 2008;47(3):401-9.
3. Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol. 2010;17(7):1055-65.
4. Song JY, Moseley MA, Burton RL, Nahm MH. Pneumococcal vaccine and opsonic pneumococcal antibody. J Infect Chemother. 2013;19(3):412-25.
5. Abbas AK, Lichtman AH, Pillai S. Effector mechanisms of humoral immunity. In: Schmidtt W, editor. Cellular and Molecular Immunology. 6th ed. Philadelphia: Saunders; 2007. p. 321-48.
6. Underhill DM, Ozinsky A. Phagocytosis of microbes: complexity in action. Annu Rev Immunol. 2002;20(1):825-52.
7. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol. 2010;11(9):785-97.
8. Guilliams M, Bruhns P, Saeys Y, Hammad H, Lambrecht BN. The function of Fc receptors in dendritic cells and macrophages. Nat Rev Immunol. 2014;14(2):94-108.
9. Borrow R, Balmer P, Miller E. Meningococcal surrogates of protection--serum bactericidal antibody activity. Vaccine. 2005;23(17-18):2222-7.
10. Martin D, McCallum L, Glennie A, Ruijne N, Blatchford P, O'Hallahan J, et al. Validation of the serum bactericidal assay for measurement of functional antibodies against group B *meningococci* associated with vaccine trials. Vaccine. 2005;23(17-18):2218-21.
11. Balmer P, Borrow R. Serologic correlates of protection for evaluating the response to meningococcal vaccines. Expert Rev Vaccines. 2004;3(1):77-87.
12. Groves E, Dart AE, Covarelli V, Caron E. Molecular mechanisms of phagocytic uptake in mammalian cells. Cell Mol Life Sci. 2008;65(13):1957-76.
13. Ison CA, Anwar N, Cole MJ, Galassini R, Heyderman RS, Klein NJ, et al. Assessment of immune response to meningococcal disease: comparison of a whole-blood assay and the serum bactericidal assay. Microb Pathog. 1999;27(4):207-14.

14. Tan TQ. Pediatric invasive pneumococcal disease in the United States in the era of pneumococcal conjugate vaccines. Clin Microbiol Rev. 2012;25(3):409-19.
15. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect. 2014;3(6):e45.

Tables

Table 1. Required concentrations for the diluted bacterial and whole-blood suspensions in function of the desired multiplicity of infection (MOI).

		Concentrations for desired MOI (CFU/ml or leukocytes/ml)				
		1	0.5	0.1	0.05	0.01
Bacteria	Diluted (to add)	1.25×10^7	6.25×10^6	1.25×10^6	6.25×10^5	1.25×10^5
	Final (Assay)	2.5×10^6	1.25×10^6	2.5×10^5	1.25×10^5	2.5×10^4
Leukocytes (Whole-Blood)	Diluted (to add)			6.25×10^6		
	Final (Assay)[1]			2.5×10^6		

[1] For a total of 5×10^5 leukocytes in a final volume of 200 µl.

Table 2. Volumes of the components to be added to the OPA microtubes, in respective order.

	Final test serum concentration			
	40 %	20 %	10 %	5 %
Diluted whole-blood *6.25 x 10^6 leukocytes/ml*	80 µl	80 µl	80 µl	80 µl
Test serum *(naive, placebo or immunized)*	80 µl	40 µl	20 µl	10 µl
Complete RPMI medium	0 µl	40 µl	60 µl	70 µl
Diluted bacteria[1]	40 µl	40 µl	40 µl	40 µl
Total volume	200 µl	200 µl	200 µl	200 µl

[1] Different multiplicity of infection ratios can be performed; see Table 1 for details on how to obtain the desired bacterial concentration.

Figures

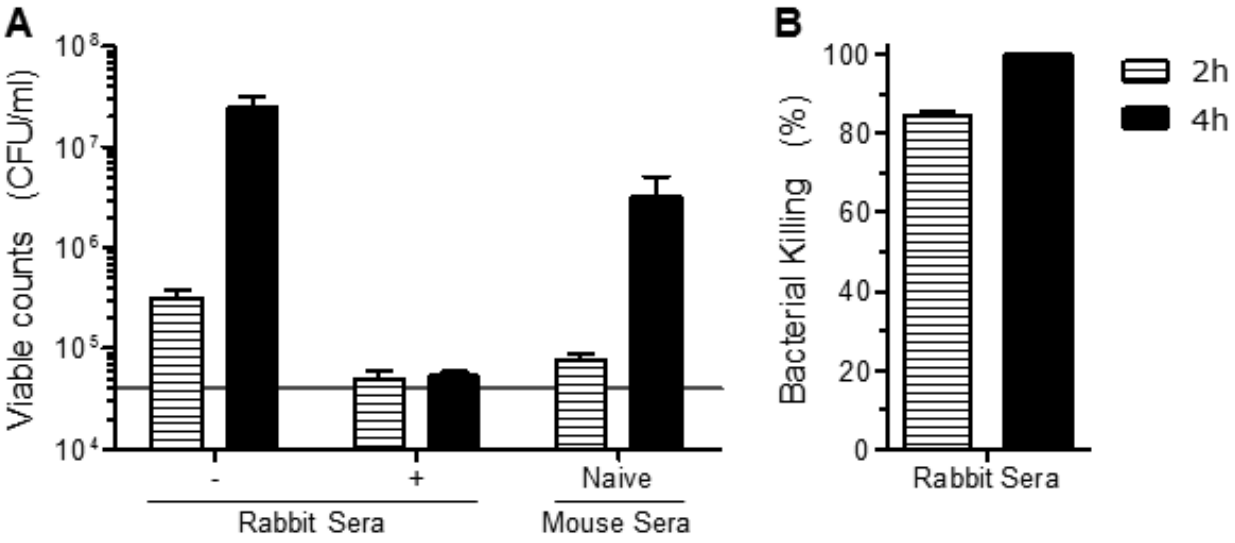


Figure 1. Optimal assay conditions for *S. pneumoniae* serotype 14 are a multiplicity of infection (MOI) of 0.01, an incubation time of 4 h, and addition of 20% of test sera.

(A) Viable counts with a MOI of 0.01 obtained at 2 h and 4 h for negative (-) and positive (+) rabbit sera (internal controls) and for a pool of naive mouse sera (at 20%). The grey line represents the bacterial inoculum value (time = 0 min). (B) Bacterial killing values at 2 h and 4 h were calculated for positive rabbit control serum (using matching negative rabbit serum for the formula). The OPA was performed at a serum concentration of 20% and a MOI of 0.01. Results are expressed as mean ± SEM from at least 3 independent experiments.

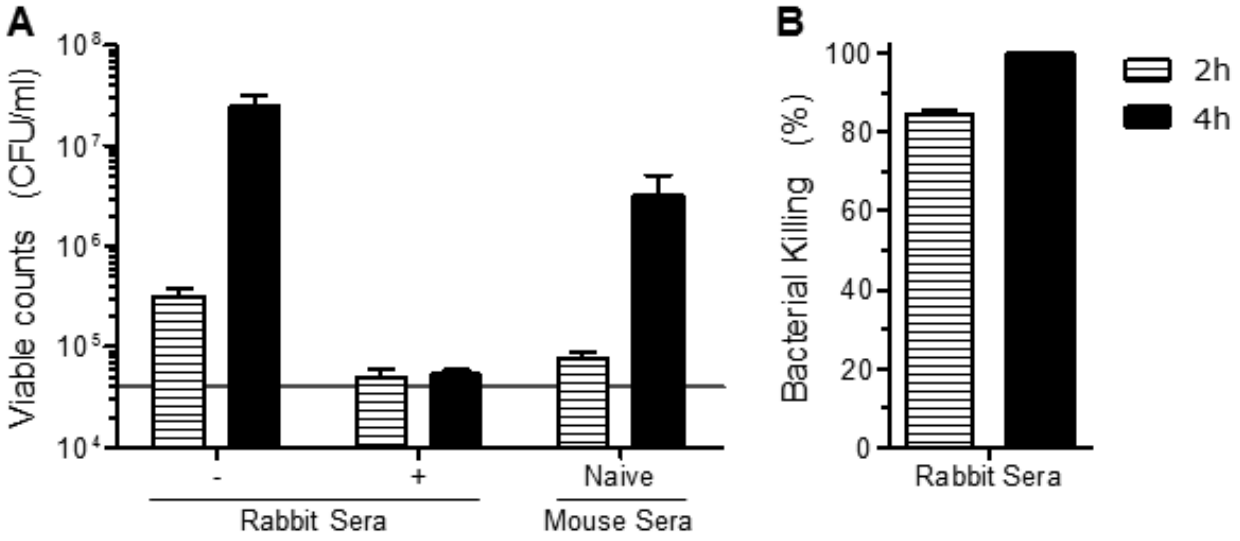


Figure 2. Optimal assay conditions for *S. suis* serotype 2 are a multiplicity of infection (MOI) of 0.1, an incubation time of 2 h, and addition of 40% of test sera.

(A) Viable counts with a MOI of 0.1 obtained at 2 h and 4 h for negative (-) and positive (+) rabbit sera or matched positive (anti-*S. suis*) and negative monoclonal antibodies (mAb), used as internal controls or for a pool of naive mouse sera (at 40%). The grey line represents the bacterial inoculum value (time = 0 min). (B) Bacterial killing values at 2 h and 4 h were calculated for positive rabbit control serum and for positive control mAb (using matching negative rabbit serum or negative mAb for the formula, respectively). The OPA was performed at a serum/mAb concentration of 40% and a MOI of 0.1. Results are expressed as mean ± SEM from at least 3 independent experiments.

ARTICLE VI

Protection Against *Streptococcus suis* Serotype 2 Infection Using a Capsular Polysaccharide Glycoconjugate Vaccine

---

Guillaume Goyette-Desjardins [1], Cynthia Calzas [1], Tze Chieh Shiao [2], Axel Neubauer [3], Jennifer Kempker [3], René Roy [2], Marcelo Gottschalk [4], and Mariela Segura [1]

[1] Laboratory of Immunology, Faculty of Veterinary Medicine, University of Montreal, St-Hyacinthe, Quebec, Canada

[2] Pharmaqam, Department of Chemistry, Université du Québec à Montréal, Montreal, Quebec, Canada

[3] Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri, USA

[4] Laboratory of research on *Streptococcus suis,* Faculty of Veterinary Medicine, University of Montreal, St-Hyacinthe, Quebec, Canada

Publié dans *Infection and Immunity*. **2016**. 84(7):2059-2075.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai participé activement à la conception de l'étude, à la réalisation des expériences (75%), à l'analyse des résultats et à l'écriture de l'article.

Abstract

Streptococcus suis serotype 2 is an encapsulated bacterium and one of the most important bacterial pathogens in the porcine industry. Despite decades of research for an efficient vaccine, none is currently available. Based on the success achieved with other encapsulated pathogens, a glycoconjugate vaccine strategy was selected to elicit opsonizing anti-capsular polysaccharide (CPS) IgG antibodies. In this work, glycoconjugate prototypes were prepared by coupling *S. suis* type 2 CPS to tetanus toxoid, and the immunological features of the post-conjugation preparations were evaluated *in vivo*. In mice, experiments evaluating three different adjuvants showed that CpG ODN induces very low levels of anti-CPS IgM antibodies, while the emulsifying adjuvants STIMUNE® and TiterMax Gold® both induced high levels of IgGs and IgM. Dose-response trials comparing free CPS with the conjugate vaccine showed that free CPS is non-immunogenic independently of the dose used, while 25 µg of the conjugate preparation was optimal in inducing high levels of anti-CPS IgGs post-boost. With an opsonophagocytosis assay using murine whole-blood, sera from immunized mice showed functional activity. Finally, the conjugate vaccine showed immunogenicity and induced protection in a swine challenge model. When conjugated and administered with emulsifying adjuvants, *S. suis* type 2 CPS is able to induce potent IgM and isotype-switched IgGs in mice and pigs, yielding functional activity *in vitro* and protection against a lethal challenge *in vivo*, all features of a T-dependent response. This study represents a proof of concept for the potential of glycoconjugate vaccines in veterinary medicine applications against invasive bacterial infections.

Introduction

Streptococcus suis is a Gram-positive encapsulated bacterium andone of the most important bacterial pathogens in the porcine industry, resulting in important economic losses (1). To date, 35 *S. suis* serotypes have been described based on the capsular polysaccharide (CPS) antigenic diversity. *S. suis* serotype 2 is considered the most virulent and most frequently isolated serotype from clinical samples and associated with disease in pigs in most countries (2). *S. suis*, mainly serotype 2, is also an important emerging zoonotic agent for humans in close contact with pigs or pig-derived products (2). The natural habitat of *S. suis* is the upper respiratory tract of pigs, more particularly the tonsils and nasal cavities, as well as the genital and digestive tracts (3). Of the various manifestations of the disease, septicemia and meningitis are by far the most striking, but other clinical outcomes can also be observed (4). Although the incidence of disease in swine varies over time and is generally less than 5%, mortality rates can reach 20% in the absence of treatment (5). Affected animals are generally between 5 and 10 weeks of age, but infections have also been reported from newborn piglets to 32 week-old pigs (3).

The thick surface-associated *S. suis* CPS confers the bacteria protection against the immune system, notably by resisting phagocytosis (6). As with most extracellular encapsulated bacteria, protection against *S. suis* is likely mediated by opsonizing antibodies which induce bacterial clearance by opsonophagocytosis. Anti-CPS antibodies have previously been demonstrated to be protective against *S. suis* serotype 2 infection following passive immunizations (7-9).

Research has been ongoing for years in the hope of developing an efficient commercial vaccine to protect post-weaning pigs against *S. suis* disease. Yet, to our knowledge, no such vaccine is available. Commercial or autogenous bacterins, which are suspensions of heat-killed or formalin-killed bacteria, are used in the field with limited success (1, 10-13). Other strategies have been experimentally tested such as live strains and sub-unit vaccines. The use of live avirulent strains gave inconsistent results and may present some safety concerns (zoonosis) (11, 14, 15). After several years of research, there is still no proven and

commercially available protein-based subunit vaccine using well characterized virulence factors and/or protective antigens (15). While some of these candidates failed, others are promising and still early in their clinical investigations. Being a pathogen with a multifactorial virulence mechanism and presenting a relatively high phenotypic heterogeneity, vaccine development is a real challenge (2, 16).

As the CPS is the most external bacterial layer in contact with the host, antibodies against it are highly opsonizing and protective, as demonstrated for several encapsulated pathogens (17-20). Paradoxically, due to their carbohydrate nature, CPSs are in general considered poorly immunogenic and do not generate long-lasting adaptive immune responses. Indeed, polysaccharides, unlike proteins and peptides, are generally recognized as T cell-independent (TI) antigens, which illustrate their innate inability to stimulate helper T cells (Th) via MHC class-II signaling, resulting in low immune cell proliferation, no antibody class switching or affinity/specificity maturation, and more importantly, lack of immunological memory (21). The scientific advancement allowing for the widespread use of carbohydrate-based vaccines was the discovery that, when properly conjugated to protein carriers serving as T cell-dependent (TD) epitopes, polysaccharides become potent vaccine antigens (21). These vaccines were named ‘glycoconjugate vaccines’. Since it has been reported that anti-CPS antibodies, if produced, have a high protective potential against infection caused by *S. suis* (22, 23), an interesting strategy for the development of a *S. suis* type 2 vaccine would be the use of a glycoconjugate vaccine made of CPS coupled to an immunogenic carrier protein, such as tetanus toxoid (TT), diphtheria toxoid, cross-reactive material 197 (CRM_{197}) and many more (24). As a matter of fact, glycoconjugate vaccines are very successful in the fight against encapsulated human pathogens such as *Haemophilus influenzae* (Hiberix®), *Neisseria meningitidis* (MenACWY®), and *Streptococcus pneumoniae* (PCV13®) (20). Despite the popular use of glycoconjugate vaccines in human medicine, this strategy has been poorly developed for veterinary practice. A CPS conjugate vaccine has been suggested for veterinary use against *Actinobacillus pleuropneumoniae*, the etiological agent of porcine pleuropneumonia (25-27).

In 2010, Van Calsteren *et al.* reported the exact structure of the repeating unit for the serotype 2 CPS (28). The CPS repeating unit is composed of a unique arrangement of 1 rhamnose : 1 glucose : 3 galactoses : 1 *N*-acetylglucosamine : 1 sialic acid (also named as *N*-acetylneuraminic acid [Neu5Ac]). The sialic acid (Neu5Ac) is found to be terminal on a branch with an α2,6-linkage to a galactose. The precise knowledge of *S. suis* serotype 2 CPS structure provides the chemical bases for the construction of a glycoconjugate. Thus, the main objective of this study was to explore the feasibility/potential, immunogenicity and protection in mice and in pigs of a CPS-TT conjugate vaccine against *S. suis* serotype 2. The effect of different adjuvants on the immunological features of the antibody response against the CPS was also studied.

Material and Methods

All chemical reagents used are of ACS grade or higher, either from Sigma-Aldrich (Oakville, ON, Canada) or Acros Organics (Fisher Scientific, Ottawa, ON, Canada).

Bacterial strains and growth conditions

S. suis serotype 2 reference strain S735 (ATCC 43765) was used as the source of type 2 CPS (28), as the target strain for *in vitro* opsonophagocytic assays (OPA), and to prepare the heat-killed bacteria used to hyperimmunize mice. Isolated colonies on sheep blood agar plates were inoculated in 5 ml of Todd-Hewitt Broth (THB; Oxoid, Nepean, ON, Canada) and incubated for 8 h in a water bath at 37°C with 120 rpm agitation. Working cultures were prepared by transferring 10 µl of 8 h-cultures diluted 1:1,000 with phosphate-buffered saline (PBS) into 30 ml of THB which was incubated for 16 h. Bacteria were washed once and resuspended in PBS to obtain 5 x 10^8 CFU/ml. Heat-killed bacterial cultures were obtained as previously described (29). Briefly, overnight cultures were washed once with PBS, then resuspended in 30 ml of fresh THB. A sample was taken to perform bacterial counts on THB Agar (THA). Bacteria were immediately killed by incubating at 60°C for 45 min, then cooled on ice. Bacterial killing was confirmed by absence of growth on blood agar for 48 h. Strains used for the swine challenge model are described below.

Isolation and purification of type 2 S. suis CPS

Bacterial cultures were performed as described by Calzas *et al.* (30). CPS extraction and purification, followed by quality controls comprising protein determination by the modified Lowry protein assay kit (Pierce, Rockford, IL), nucleic acid quantification using an ND-1000 spectrometer (Nanodrop, Wilmington, DE) and 1D/2D ^{1}H nuclear magnetic resonance (NMR) analysis to ensure purity and identity were performed as described by Van Calsteren *et al*. (28).

Depolymerization of type 2 CPS

Twenty milliliters of a 2 mg/ml solution of CPS in 50 mM NH_4HCO_3 were transferred to a 50 ml conical polypropylene tube in an ice-bath. A titanium 1/8 inch microtip probe mounted on a Virsonic 600 sonicator (Virtis, Gardiner, NY) was immersed in the CPS solution, and sonication was performed at 20 kHz and 24 W for 60 min. After sonication, a sample of CPS was taken to determine the weight-average molar mass (M_w) by size-exclusion chromatography coupled with multi-angle light scattering (SEC–MALS) as previously described (31), with some modifications. Briefly, the chromatographic separation was performed with two 8 mm × 300 mm Shodex OHpak gel filtration columns connected in series (SB-806 and SB-804), preceded by a SB-807G guard column (Showa Denko, Tokyo, Japan). Elution was done at 0.5 ml/min using 0.1 M $NaNO_3$ as the mobile phase. Molar masses were determined using a Dawn EOS MALS detector (Wyatt, Santa Barbara, CA) and calculations were performed with the ASTRA software version 6.1.1.17 (Wyatt) using 11 detectors from angles 34.8° to 132.2° (detectors 5–15) for the depolymerized samples. The remaining solution of depolymerized CPS was dialyzed against water (Spectra/Por, MWCO 3,500; Spectrum Laboratories, Rancho Dominguez, CA) and lyophilized. The optimal conditions for sonication were determined in pre-tests using different time points (Fig. S1).

Mild periodate oxidation of depolymerized CPS

Depolymerized *S. suis* serotype 2 CPS (8.8 mg, 6.7 μmol) was incubated with 620 μM of sodium periodate in 1.1 ml of water in the dark with a stirring magnet at room temperature for 1 h. An excess of two equivalents of triethylene glycol per periodate was added for 1 h to consume any residual periodate. The mixture was dialyzed against water (Spectra/Por,

MWCO 1,000; Spectrum Laboratories) and lyophilized. The optimal conditions for oxidation were determined in preliminary tests (data not shown).

The degree of oxidation of the sialic acid (Neu5Ac) residues was assessed by gas chromatography (GC) analysis of the peracetylated methyl glycosides adapted from a previously described method (32). Briefly, oxidized CPS (0.4 mg) was reduced by adding 100 µl of $NaBH_4$ (10 mg/ml) in water for 1 h at room temperature. The reaction was quenched with 5% acetic acid solution in methanol and evaporated to dryness using a stream of N_2. Evaporations were repeated 3 times by the addition of 250 µl of methanol each time. The composition of the residue was determined by methanolysis. To this aim, methanol (465 µl) and acetyl chloride (35 µl), which generate HCl, were added to the residue. The solution was heated for 17 h at 75°C, evaporated to dryness, followed by addition of 500 µl of *tert*-butanol and evaporated to dryness again. The methyl glycosides were acetylated with 150 µl of pyridine and 150 µl of acetic anhydride at 100°C for 20 min. The cooled solution was partitioned with 5 ml of water and 1 ml of CH_2Cl_2. The organic layer containing the peracetylated methyl glycosides was analyzed by GC using flame ionization detection (GC-FID). GC-FID analysis was done on a Hewlett-Packard model 7890 gas chromatograph equipped with a 30-m by 0.32-mm (0.25-µm particle size) HP-5 capillary column (Agilent Technologies, Santa Clara, CA) using the following temperature program: 50°C for 2 min, an increase of 30°C/min to 150°C, then an increase of 3°C/min to 230°C, and a hold for 5 min. The temperatures of the injector and the flame ionization detector were 225°C and 250°C, respectively.

Purification of tetanus toxoid monomer

TT monomer was obtained by gel filtration chromatography before conjugation. One milliliter of a liquid preparation containing 4.5 mg/ml protein (as determined by the modified Lowry protein assay) was loaded onto a XK16-100 column filled with Superdex 200 Prep Grade (GE Healthcare Life Sciences, Uppsala, Sweden) equilibrated in PBS (20 mM $NaHPO_4$ pH 7.2, 150 mM NaCl) and eluted with the same buffer. The protein eluted from the column in two peaks: the earlier eluting peak contained oligomerized toxoid and the later eluting peak, corresponding to a M_r of 150,000, contained TT monomer. Fractions corresponding to the later

(monomer) peak were pooled, desalted against deionized water and concentrated using Centricon Plus-70 centrifugal filter device (30K Ultracel PL membrane; Millipore, Billerica, MA), then lyophilized.

Conjugation of type 2 CPS to TT by reductive amination

Periodate treated type 2 CPS (3.6 mg, 40 nmol) and purified TT monomer (3.0 mg, 20 nmol) were dissolved in 2.2 ml of 0.1 M sodium bicarbonate pH 8.1 for the 2:1 conjugate ratio. Sodium cyanoborohydride (7.5 mg, 120 μmol) was added, and the mixture was incubated at 37°C with orbital agitation for 2 days. For the 1:1 conjugate ratio, conjugation was performed in the same manner as described above except by using 1.8 mg (20 nmol) of oxidized CPS. Sodium borohydride (4.7 mg, 124 μmol) was then added to the reaction mixture to reduce any remaining free aldehyde groups. Produced 2:1 and 1:1 mixed conjugates were extensively dialyzed against water (Spectra/Por, MWCO 3,500; Spectrum Laboratories) and lyophilized. Conjugation was controlled by Gel Shift on SDS-PAGE, immunoblotting and high-performance liquid chromatography (HPLC) as described below. The conditions for conjugation by reductive amination were determined in pre-tests using different CPS to TT ratios, different % of CPS oxidation and different incubation times (data not shown).

SDS-PAGE and immunoblotting

Samples were diluted in twice concentrated SDS-PAGE loading buffer containing 2-mercaptoethanol, denatured by heating at 100°C for 5 min and separated by SDS-PAGE on 7.5% acrylamide gels. The gels were either stained with Coomassie G-250 Stain or Silver Stain Kit (Bio-Rad Hercules, CA) or transferred to nitrocellulose Western blot membrane (Bio-Rad). The membrane was then blocked with a solution of Tris-buffered saline (TBS; 10 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 2% skim milk for 1 h. The membrane was washed 3 times with TBS, and incubated for 2 h with either a mouse monoclonal antibody (mAb) against serotype 2 CPS of *S. suis* (clone Z3; (23)) diluted 1:50 or a mouse mAb against TT (clone HYB 278-01; Abcam, Toronto, ON, Canada) diluted 1:500 in blocking buffer. The membrane was then washed 3 times with TBS and incubated 1 h with a goat anti-mouse IgG+IgM (H+L) horseradish peroxidase (HRP) -conjugated antibody (Jackson Immunoresearch, West Grove, PA) either diluted 1:3,000 for CPS or diluted 1:1,000 for TT in

blocking buffer. The membrane was washed 3 times with TBS and developed with a 4-chloro-1-naphtol solution (Sigma-Aldrich).

HPLC analysis of the conjugates

HPLC analysis of the glycoconjugate preparations was done by size-exclusion chromatography. The chromatographic separation was performed with three 8 mm x 300 mm Shodex OHpak gel filtration columns connected in series (two SB-804 and one SB-803) preceded by a SB-807G guard column (Showa Denko). The glycoconjugate vaccine was eluted with 0.1 M $NaNO_3$ at a flow rate of 0.4 ml/min using a Knauer Smartline system equipped with a differential refractometer (RI) detector model 2300 and an UV detector model 2600 at a wavelength of 280 nm. The conjugate preparation (8 mg/ml solution in the mobile phase) was injected using a 50 µl injection loop. In selected experiments (see below), the fractions eluting at the void volume, that correspond to the conjugate fractions, were pooled, dialyzed against water (Spectra/Por, MWCO 12,000-14,000; Spectrum Laboratories) and lyophilized. This corresponds to the 2:1 fractionated conjugate.

Mouse immunizations

All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals by the Animal Welfare Committee of the University of Montreal. Five to 6 week-old C57BL/6 female mice (Charles River, Wilmington, MA), largely used for studies on *S. suis* pathogenesis (17, 33-35), were immunized subcutaneously with different doses of the *S. suis* CPS conjugate preparations (see Results) in 0.1 ml PBS on day 0 and boosted on day 21. In a first set of experiments aimed to compare different adjuvants, 3 groups (n = 10) received 25 µg of the 2:1 mixed conjugate dissolved in PBS adjuvanted with either 20 µg of CpG oligodeoxyribonucleotide (ODN) 1826 (InvivoGen, San Diego, CA), STIMUNE® (Prionics, La Vista, NE) or TiterMax Gold® (CytRx Corporation, Norcross, GA) following manufacturers' recommendations. Three placebo groups (n = 5) received only PBS adjuvanted as described above. In a second set of experiments, a dose-response study was performed using groups of mice (n = 8) immunized with either 1, 2.5, 5 or 25 µg of the 2:1 mixed conjugate emulsified 1:1 (v/v) with TiterMax Gold®. Mice (n = 8)

immunized with similar doses of free (unconjugated) depolymerized CPS emulsified with TiterMax Gold® were included for comparison purposes. A placebo group (n = 5) was also included. In a third set of experiments, to compare the efficiency of different conjugates, groups of mice (n = 10) received either 25 μg of the 1:1 mixed conjugate, 2:1 fractionated conjugate or a free (unconjugated) mixture of 2 CPS: 1 TT. All preparations were emulsified with TiterMax Gold® and a placebo group was also included.

In all experiments, to follow antibody responses, mice were bled (10 μl) weekly on days -1, 7, 14, 21, 28, 35, and 41 post-immunization by the tail vein. Diluted blood was directly used in the ELISA test as described below. At day 42 post-immunization mice were humanely euthanized and sera collected and frozen at -80°C for ELISA Ig titration and isotyping, and for OPA analyses (see below).

Immunization and challenge of pigs

Animals were treated in accordance with the ethical guidelines of the Institutional Animal Care and Use Committee of Boehringer Ingelheim Vetmedica, Inc. A total of 44 3-week-old piglets (±5 days) were obtained from a commercial herd in Nebraska, USA, for this study. Tonsil swabs from the study piglets were negative by *S. suis* serotype 2-PCR prior to study initiation (2). The piglets were blocked by litter and then randomly assigned to one of four groups by a bio-statistician using SAS® version 9.3: group 1, n = 14; group 2, n = 10, group 3, n = 15; and group 4, n = 5. Groups 1-4 were commingled until group 4 (strict control) was removed at study day 35. Blood samples were collected on study days 0, 21 and 34 for determination of serum antibody levels.

The vaccines and the placebo were adjuvanted with STIMUNE® according to the manufacturer's instructions. The piglets were injected intramuscularly twice at a 3-week interval (study day 0 and 21) with 2 ml of the respective vaccine or placebo: group 1 was vaccinated with formalin inactivated, adjuvanted *S. suis* type 2 culture containing 2 x 10^{10} bacteria (isolate 3977B; a virulent field isolate of *S. suis* serotype 2 previously used in a bacterin efficacy study; Neubauer, unpublished), group 2 was injected with the adjuvanted 2:1

mixed conjugate vaccine containing either 85 µg (1st vaccination) or 56 µg (second vaccination) of the antigen, group 3 was given 2 ml of adjuvanted PBS.

On day 36, groups 1-3 were challenged intraperitoneally with 2 ml (3×10^9 CFU/dose) of a late exponential *S. suis* type 2 culture grown in THB with 5% fetal bovine serum. The animals were challenged with isolate ATCC 700794, which is the established challenge strain in the model used. Following challenge, pigs were monitored daily over a period of seven days for the presence of clinical signs. The individuals observing the animals were blinded to the treatments. Assessed were behavior, including those indicating functional alteration of the central nervous system (CNS), and locomotion. The observations for general behavior were numerically scored as follows: 0 = physiological, 1 = depression, 2 = apathy. Observations for locomotion were scored as 0 = physiological, 1 = slightly to moderately lame, 2 = severely lame/ reluctant to stand, 3 = animal partially /completely down; animals can rise, but lie down again within 10 seconds. CNS signs were scored as 0 = absent and 1 = present. In accordance with the humane endpoints defined in the animal use protocol as well as 9CFR 117.4 (36), animals being unresponsive to stimuli, animals exhibiting CNS signs as well as animals that were assigned a lameness score of "3" were humanely euthanized. At study day 43, all remaining animals were humanely euthanized.

All animals found dead or that had been euthanized were necropsied. Meninges, pericardium, and joint swabs were collected and streaked out on Columbia blood agar plates. Alpha hemolytic colonies present on the plates following overnight incubation were tested with type 2 antisera and, as deemed necessary, by *S. suis* type 2 PCR. Gross pathology observations were recorded for the thoracic cavity (presence of fibrin, fluid, congestion and pericarditis), and for the joints (presence of fibrin and fluid). The individuals conducting the necropsies were blinded to the treatments.

Control mouse antiserum

Hyperimmune mice (n = 6) were obtained by repeated immunization of 5 week-old female C57BL/6 mice with 7.5×10^8 CFU/ml heat-killed *S. suis* serotype 2 strain S735 in

THB by intraperitoneal injection on days 0, 7, 21, and 28. On day 42, serum was collected, pooled, aliquoted, and stored at -80°C.

Measurement of antibodies against type 2 S. suis CPS and TT

To measure specific antibodies, 200 ng of either native *S. suis* serotype 2 CPS or TT in 0.1 M $NaCO_3$, pH 9.6, were added to wells of an ELISA plate (Nunc-Immuno Polysorp, Canadawide Scientific, Toronto, ON, Canada). After overnight coating at 4 °C, plates were washed with PBS containing 0.05% (v/v) Tween 20 (PBST) and blocked by the addition of PBS containing 1% (w/v) of BSA (HyClone, Logan, UT) for 1 h. After washing, mouse blood or mouse/porcine serum samples diluted in PBST were added to the wells for 1 h. After washing, the plates were incubated for 1 h with a HRP-conjugated isotype specific antibody diluted in PBST as described below. The enzyme reaction was developed by addition of 3,3',5,5'-tetramethylbenzidine (TMB; InvitroGen, Burlington, ON, Canada), stopped by addition of 0.5 M H_2SO_4, and the absorbance was read at 450 nm with an ELISA plate reader.

To follow the kinetics of total (IgG+IgM) antibody responses to CPS and TT, mouse blood collected from the tail vein was diluted 1:100 or 1:20,000, respectively. Dilution optimization had previously been conducted (data not shown). HRP-conjugated goat anti-mouse IgG+IgM (H+L) at a dilution of 1:2,500 (Jackson Immunoresearch) was used as detection antibody.

To perform the titration of mouse Ig isotypes, day 42-serum was serially diluted (two-fold) in PBST, and antibodies were detected using either HRP-conjugated goat anti-mouse IgG+IgM as aforementioned, goat anti-IgM diluted 1:1000, goat anti-IgG1, goat anti-IgG2b, goat anti-IgG2c or goat anti-IgG3 diluted 1:400 (Southern Biotech). For porcine serum, two-fold serial dilutions were performed in PBST and antibodies were detected using HRP-conjugated goat anti-swine total Ig [IgG+IgM] diluted 1:4,000 (Jackson Immunoresearch). To detect porcine IgG subclasses, unconjugated mouse anti-swine IgG1 or mouse anti-swine IgG2 (AbD serotec, Raleigh, NC) diluted 1:250 was added followed by incubation with HRP-conjugated goat anti-mouse secondary antibody. For both, mouse and pig serum titration, the reciprocal of the last serum dilution that resulted in an optical density ($OD_{450\ nm}$) equal or

lower of 0.2 (as a pre-established cutoff for comparison purposes) was considered the titer of that serum. For representation purposes, negative titers (≤ cutoff) were given an arbitrary titer value of 10.

To control inter-plate variations, an internal reference positive control was added to each plate. For titration of mouse antibodies, this control was a pool of sera from hyper-immunized mice (produced as described above). For titration of pig antibodies, this control was a serum of a pig hyper-immunized with 10^8 CFU of a killed suspension of *S. suis* serotype 2. Reaction in TMB was stopped when an $OD_{450\ nm}$ of 1 was obtained for the positive internal control. Optimal dilutions of the coating antigen (CPS or TT), the positive internal control sera and the HRP-conjugated anti-mouse or anti-pig antibodies were determined during preliminary standardizations.

Opsonophagocytosis Assay

Blood was collected by intracardiac puncture from naïve C57BL/6 mice, treated with sodium heparin, then diluted to obtain 6.25 x 10^6 leukocytes/ml in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine and 50 µM 2-mercaptoethanol. All reagents were from Gibco (InvitroGen, Burlington, ON, Canada). All blood preparations were kept at room temperature. Using washed bacterial cultures grown as described above, final bacterial suspensions were prepared in complete cell culture medium to obtain a concentration of 1.25 x 10^6 CFU/ml. The number of CFU/ml in the final suspension was determined by plating samples onto THA using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA). All bacterial preparations were kept on ice. Diluted whole blood at 5 x 10^5 leukocytes was mixed with 5 x 10^4 CFU of *S. suis* (multiplicity of infection [MOI] of 0.1) and 40% (v/v) of serum from naïve or vaccinated mice in a microtube to a final volume of 0.2 ml. The tube tops were pierced using a sterile 25G needle, then the microtubes were incubated for 2 h at 37°C with 5% CO_2, with gentle manual agitation every 20 min. After incubation, viable bacterial counts were performed on THA using an Autoplate 4000 Automated Spiral Plater. Tubes with addition of naive rabbit serum or rabbit anti-*S. suis* type 2 strain S735 serum (37) were used as negative and positive controls, respectively. The % of bacterial killing was determined using the following formula: % Bacteria killed = [1-

(bacteria recovered from sample tubes/ bacteria recovered from negative control tubes with naïve mouse sera)] × 100. Final OPA conditions were selected based in several pre-trials using different incubation times and MOIs (38).

Statistical analyses

All data are expressed as means ± standard errors of the means (SEM). Data were analyzed for significance using analysis of variance (ANOVA) from SigmaPlot version 11.0, except for the survival curves analysis, which was performed using the log-rank test from GraphPad version 5.01. Significance is denoted in the figures as follows: *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

Summaries of and data analyses for the pig study were conducted by a bio-statistician using SAS® Version 9.3. Clinical observations (death, lameness, CNS signs, and behavioral changes) were summarized as frequencies by day and treatment. Incidence of normal versus not normal for each characteristic were analyzed where appropriate using the GLIMMIX procedure of SAS with binomial error and logit link. The model included the fixed effect of treatment and the random effects of litter and residual. In addition, the proportion of the observations for each animal that were not normal was analyzed. Prior to analysis, the proportion was transformed using the arcsine square root transformation. The mixed model included the fixed effect of treatment and the random effects of litter and residual. Comparisons of interest include the following and were evaluated using a two-sided test with alpha = 0.05: Groups 1 (bacterin) and 2 (conjugate) vs. 3 (protection provided against challenge with isolate ATCC 700794).

Results

Preparation of the conjugate vaccines

Using highly purified CPS from *S. suis* type 2 containing less than 1% w/w of proteins or nucleic acids (as previously described by Calzas *et al.* (30)), we investigated whether conjugation to a carrier protein, such as TT, would circumvent the TI antigenicity of the CPS and instead induce a TD protective humoral response in animals.

Due to its high M_w, found to be between 410,000-480,000 Da by SEC-MALS (28, 30), the native polysaccharide of *S. suis* type 2 must first be depolymerized into smaller fragments. This reduces polydispersity and yields higher ratios of polysaccharide over protein following coupling. To perform this depolymerization, we opted for ultrasonic irradiation as described by Szu *et al.* (39). By monitoring CPS M_w of samples by SEC-MALS during pre-tests, it was shown that depolymerization plateaued after 45 min of sonication (Fig. S1). Based on these observations, we selected a depolymerization time of 60 min of sonication at which two different lots were produced with reproducible results giving an average M_w of 115,000 Da (113,000-118,000 Da; Table S1). ^{1}H NMR investigations of these two lots found no structural alteration of the polysaccharide other than the depolymerization itself (data not shown). These depolymerized CPSs were used in the subsequent preparation of the conjugate vaccine formulations.

The presence of sialic acid (Neu5Ac) as a constituent in the repeating unit sequence of *S. suis* serotype 2 CPS granted the use of mild conditions in order to achieve an oxidative cleavage between C8-C9 of the glycerol side-chain, thus leaving free terminal aldehydes as reacting groups for subsequent conjugation to TT by reductive amination (40). To preserve CPS immunogenicity, a 10% level of oxidation was targeted, leaving 90% of all Neu5Ac untouched. For a ~115,000 Da long CPS, this resulted in an average of 9 oxidized Neu5Ac per chain. Following pre-tests, 0.1 equivalent of sodium periodate per Neu5Ac was selected, and the two different CPS lots were oxidized. Reproducible oxidation levels at C8 of 9.2-9.4% were obtained as determined by GC-FID analysis of the peracetylated methyl glycosides. No oxidation at C7 was observed under these conditions. ^{1}H NMR investigations found no other structural modification of the polysaccharide (data not shown).

The depolymerized-oxidized CPS and purified TT monomer were conjugated at a molar ratio of 2 chains of CPS:1 TT or at a molar ratio of 1:1 by reductive amination (41). The optimal incubation time for conjugation was found to be 2 days during pre-tests (data not shown). After incubation, remaining aldehyde groups were reduced by the addition of sodium borohydride. Reagents were then eliminated from the conjugate mixes by extensive dialysis

against water. ***Gel shift, Western blot and HPLC analysis confirmed successful conjugation of CPS to TT***

The presence of conjugates in the different preparations was verified by Gel shift, Western blot experiments (Fig. 1) and by HPLC analysis (Fig. 2). For the Gel shift experiments, both Coomassie Blue (Fig. 1A) and Silver staining (Fig. 1B) showed a considerable shift from the purified TT monomer at 150 kDa (lane 2) to a thick band of over 250 kDa in the conjugates (lanes 3-4). This shift resulted from the covalent addition of a random number of 115 kDa CPS chains to the protein. Interestingly, the selected silver stain kit included one step of dichromate oxidation favoring a higher intensity signal from glycoproteins (42). Accordingly, a strong reaction was observed with the conjugate preparations (Fig. 1B, lanes 3-4). Neither Coomassie Blue (Fig. 1A), Silver staining (Fig. 1B) nor Western Blot using an anti-CPS mAb (Fig. 1C) revealed any band for the depolymerized CPS included as a control in all gels (lane 5), illustrating its weak capacity to migrate through the gels under these assay conditions. As such, positive signal observed for the bands >250 kDa when revealing using an anti-CPS mAb definitely proved the covalent nature of the linkage between CPS and TT in the conjugates (Fig. 1C, lanes 3-4). Control staining using an anti-TT mAb (Fig. 1D) shows that the epitope to which the mAb binds was preserved in the conjugates. Preservation of TT antigenicity is essential since it is the key mechanism allowing for the production of a T-dependent anti-CPS humoral response. It should be noted that differences in signal intensities between the 2:1 and 1:1 mixed conjugates (Fig. 1, lanes 3-4) are likely related to the total amounts of protein content (4.5 µg *vs.* 6.3 µg, respectively) within the 10 µg loaded sample per lane.

HPLC analysis (Fig. 2) showed the elution of the conjugate (> 250 kDa), of free CPS (100 kDa) and of free TT (150 kDa). By integrating $UV_{280\ nm}$ signal from the chromatograms, it was estimated that 48 ± 6 % (mean ± SD) of the protein content from the mixture is indeed found in the conjugate fraction. Taken together, Gel shift and Western blot experiments combined with HPLC analysis of the two conjugate samples revealed the presence of conjugates in the 2 CPS:1 TT and 1:1 preparations.

Emulsifying adjuvants present higher immunomodulatory properties than CpG ODN for a polysaccharide antigen

Using the 2:1 mixed conjugate, optimization of the immunization protocol was performed in a murine model using inbred C57BL/6 mice. The performance of three different adjuvants was compared. CpG ODN is a synthetic version of a bacterial oligonucleotide with unmethylated CpG motifs and acts as a Toll-like receptor 9 (TLR9) ligand with immunostimulatory properties toward a Th1 response (43). STIMUNE® (Specol) is a water-in-oil adjuvant composed of purified and defined mineral oil (Markol 52) with Span 85 and Tween 85 as emulsifiers (44). It has been used as a good alternative to Freund's adjuvant for weak immunogens in animals, such as mice and pigs (45). TiterMax Gold® is also a water-in-oil adjuvant consisting of squalene as a metabolizable oil, sorbitan monooleate 80 as an emulsifier and CRL8300 (a patented block copolymer) and microparticulate silica as stabilizers (44). TiterMax Gold® has been suggested as a superior alternative to Freund's adjuvant providing comparable titers with fewer injections and less undesired reactivity in mice (46).

Based on the literature (47), a dose of 20 μg of CpG was chosen to be added for adjuvanting. In parallel, conjugates were emulsified with recommended ratios of 4 parts aqueous antigen per 5 parts adjuvant for STIMUNE®, or 1:1 for TiterMax Gold®. Doses of 25 μg of the 2:1 mixed conjugate vaccine for each adjuvant were administered to mice on days 0 and 21. The kinetics of total Ig[G+M] antibody responses against CPS or TT were followed weekly from tail vein blood samples (Fig. 3). Overall, CpG ODN 1826 (Fig. 3A) gave the lowest anti-CPS and anti-TT responses. Also, anti-CPS Ig isotyping showed a strict IgM isotype response (data not shown). In contrast, STIMUNE® (Fig. 3B) and TiterMax Gold® (Fig. 3C) gave comparable strong anti-CPS and anti-TT total Ig[G+M] responses. Furthermore, anti-CPS Ig isotype switching was observed with both emulsifying adjuvants (see below). Albeit a memory antibody response against TT was observed with all three adjuvants, STIMUNE® and TiterMax Gold® induced faster and higher anti-CPS antibody levels after boost, suggesting that generation of immunological memory against the CPS antigen is favored by these emulsifying adjuvants. Finally, it should be noted that all placebo

mice, injected only with PBS and adjuvant, did not produce any non-specific antibody response (Data not shown).

As TiterMax Gold® is recognized as one of the best adjuvants for mice (48, 49), it was selected for further immunizations with this species. For its response comparable to TiterMax Gold® and its previous evaluation in large animals (49, 50), STIMUNE® was selected as the adjuvant for immunization of pigs.

While the free polysaccharide is non-immunogenic, a dose-response effect on antibody levels is observed with the conjugate vaccine

Using TiterMax Gold® as the adjuvant, mice were immunized on days 0 and 21 with doses of 1, 2.5, 5 or 25 μg of the 2:1 mixed conjugate to evaluate the dose-response effect on antibody production. Groups of mice were also immunized with different doses of *S. suis* serotype 2 free (unconjugated) CPS to assess if it could be immunogenic by itself when adjuvanted with TiterMax Gold®.

Even at a high dose (25 μg) of free CPS, no significant total Ig[G+M] primary or memory antibody responses were observed throughout the immunization period (Fig. 4). In contrast, a dose-response effect was observed when mice were immunized with the 2:1 mixed conjugate, with the 25 μg dose yielding the highest total Ig[G+M] anti-CPS antibody response as measured on weekly collected blood samples.

Conjugation of S. suis type 2 CPS to TT induces antibody isotype switching in mice

Not only a stronger response following boost (as illustrated in Figs. 3 and 4), but also antibody isotype switching are good indicators of conjugate immunogenicity and ability to induce a T cell-dependent response. As such, titers of the different anti-CPS antibody isotypes were determined in mice immunized with 25 μg of the 2:1 mixed conjugate vaccine adjuvanted with TiterMax Gold®. As shown in Fig. 5, not only strong IgM titers, but also high levels of all IgG subclasses were observed including IgG1, IgG2b, IgG2c and IgG3 specific for the CPS antigen. To evaluate if isotype switching was dependent on the adjuvant, serum samples of mice immunized with 25 μg of the 2:1 mixed conjugate adjuvanted with STIMUNE® were analyzed. STIMUNE® also induced isotype switching in mice; however,

levels were lower and profiles differed from those observed with TiterMax Gold®, with no production of the IgG2c and IgG3 subclasses (Fig. 6A).

In order to determine the effect of CPS to TT ratio on the conjugate immunogenicity, another mixed conjugate, this time using a ratio of 1 CPS: 1 TT was prepared (displayed in Fig. 1) and emulsified in TiterMax Gold®. Immunized mice showed similar IgM titers, reduced (but not significantly different) IgG1 and IgG3 titers, and significantly lower IgG2b titers ($P < 0.01$) than those induced by the 2:1 mixed conjugate in TiterMax Gold® (Fig. 5). Interestingly, the 1:1 mixed conjugate failed to induce significant titers of the IgG2c subclass.

In order to prove that the observed immunogenicity was in fact due to the conjugate present in the vaccine formulation, and not only due to remaining free CPS and TT, two additional controls were included in the study. A first control was the HPLC-isolated specific fraction corresponding to the conjugate from the 2:1 mixed conjugate (2:1 fractionated conjugate). The second control was a mixture of free CPS and free TT in the same ratio of 2:1 as before conjugation. In general, no major differences were observed between the 2:1 mixed conjugate and the specific 2:1 fractionated conjugate, yet, higher titers of IgG1, IgG2b, and IgG3 were observed with the later preparation (Fig. 5, $P < 0.01$). In contrast, the mixture of unconjugated CPS and TT gave a strong IgM titer but very low titers of IgG1 compared to the 2:1 mixed conjugate ($P < 0.01$). In addition, no production of IgG2c subclass was observed in mice immunized with this control unconjugated preparation.

Finally, the control hyperimmune sera (from mice repeatedly injected with heat-killed bacteria) resulted in a high titer of IgM, production of IgG2b and IgG2c but absence of IgG1 and IgG3 subclasses against the CPS antigen (Fig. 5). Similar results were obtained when mice were hyperimmunized with heat-killed bacteria adjuvanted in TiterMax Gold® (data not shown).

Functional activity of antibodies

Protective capacity of sera from immunized mice was evaluated using an OPA test, a recognized correlate of immunity for encapsulated Gram-positive bacteria, such as *S.*

pneumoniae (51). Instead of using a cell line or a single cell type, the OPA was standardized using whole blood from naïve mice. This model takes into account all blood leukocytes and thus represents a more realistic model of the complex interactions between all immune cells and the bacteria during a systemic infection, as is the case for *S. suis*. As shown in Fig. 7, sera from mice immunized with the 2:1 mixed conjugate adjuvanted with TiterMax Gold® induced high bacterial killing levels ranging from 64-77 %. Sera from mice immunized with the 2:1 fractionated conjugate gave higher, but not significantly different, bacterial killing values ranging from 74-98 % (Fig. 7). The effect of the adjuvant was also evaluated in the OPA test; sera from mice immunized with the 2:1 mixed conjugate adjuvanted with STIMUNE® induced bacterial killing levels ranging from 39-74 % (Fig. 6B), which were not significantly different from those induced by TiterMax Gold® ($P > 0.05$). When the OPA was performed using sera from mice immunized with unconjugated CPS and TT mixture, significant lower values (between 0-47 %) of bacterial killing were observed compared to conjugates (Fig. 7; $P < 0.001$). Finally, pooled sera from mice hyperimmunized with killed whole cell preparations gave bacterial killing values highly similar to those of the unconjugated mixture (Fig. 7; $P > 0.05$).

Immunogenicity and protection in pigs

Based on previous results, the 2:1 mixed conjugate was selected to evaluate the immunogenicity and protection in the *S. suis* natural host: the pig. The adjuvant STIMUNE® was chosen as it had been previously included in *S. suis* bacterin-based vaccines (12, 52). The performance of the conjugate was compared to that of a *S. suis* type 2 bacterin adjuvanted with STIMUNE®. To this end, pigs were injected intramuscularly twice at a 3-week interval and serum samples were collected on days 0, 21, and 34 for titration and isotyping of anti-CPS antibodies (Fig. 8A). On day 21 post-immunization, total Ig[G+M] anti-CPS titers induced by the 2:1 conjugate vaccine were significantly higher ($P < 0.01$) than those of the placebo and bacterin. After boosting, on day 34 post-immunization, an increase in anti-CPS titers was observed for both vaccinated groups compared to day 21. However, only the titers from pigs vaccinated with the 2:1 mixed conjugate were significantly higher than the placebo control group ($P < 0.001$). Titers of the different swine IgG subclasses, namely IgG1 and IgG2, were also assayed post-boost injection (day 34). Forty percent of pigs immunized with the 2:1

mixed conjugate showed significant levels of anti-CPS IgG1 subclass (Fig. 8A). No switch to the IgG2 subclass was observed (data not shown). In contrast, vaccination of pigs with the bacterin failed to induce anti-CPS Ig class switch (Fig. 8A and data not shown).

On study day 36, pigs were challenged intraperitoneally with a dose of 3 x10^9 CFU of ATCC 700794, a virulent *S. suis* serotype 2 strain. Most pigs in the placebo group died during the systemic phase of *S. suis* infection, reaching a mortality of 86.7%. In contrast, pigs immunized with the bacterin or the 2:1 mixed conjugate both showed mortality of 28.6% and of 30.0%, respectively. Analysis of survival curves (Fig. 8B) showed a significant difference as soon as day 3 between both immunized groups and the placebo group (P = 0.009). Protection induced by the 2:1 mixed conjugate was similar to that of the control type 2 bacterin during the systemic phase of a *S. suis* challenge infection in pigs.

Pigs were also monitored for clinical signs (behavior, locomotion problems or CNS signs) for seven consecutive days after challenge. In 31.6% of all observations for the bacterin-vaccinated group and in 28.1% of all observations for the conjugate-vaccinated group abnormal behavior was observed. This was significantly lower compared to the findings in the placebo group, in which 90.7% of the observations revealed abnormal behavior (Table 1, adjusted P value < 0.05). Lameness was observed in 26.3% of all observations for the bacterin-vaccinated group and in 33.5% of all observations for the conjugate-vaccinated group compared to 89.3% for the placebo group (Table 1, adjusted P value < 0.05). These differences were also observed when the distribution of clinical scores was analyzed daily for each group (data not shown). CNS signs were observed only in few pigs and no statistically significant differences were observed between the three challenged groups (Table 1). This can be explained by the fact that animals were observed for only a 7 day-period post-challenge, as the study design mainly focused on the systemic phase of the disease. All pigs found dead as well as all euthanized pigs were necropsied. The frequency of gross lesions in the thoracic cavity (i.e. fibrin, excess fluid, pericarditis) or in the joints was overall reduced in vaccinated animals compared to the placebo group. Yet observed differences did not reach statistical significance (Table S2). The conjugate vaccine significantly reduced the challenge strain recovery from joint swabs (P < 0.01). The *S. suis* challenge strain was also less frequently

isolated from the meningeal and pericardial swabs compared to the placebo group, yet differences were not statistically different (Table S3).

Discussion

While glycoconjugate vaccines (made from CPS conjugated to an immunogenic protein carrier) are very effective in the fight against encapsulated bacteria in human medicine, so far no such vaccine is available for swine veterinary practice. Although some conjugates have previously been suggested for veterinary medicine use (25-27, 53), so far none have been marketed. For the first time, we report the feasibility of a glycoconjugate vaccine to protect pigs from *S. suis* type 2 infections. This glycoconjugate, made with type 2 CPS coupled to TT, is immunogenic in mice and pigs by inducing production of IgG antibodies, which are functional *in vitro* and protective *in vivo*.

Capsular polysaccharides are considered TI antigens as they are in general unable to stimulate MHC class II-dependent Th cell help (30), which results in poor immunogenicity. In contrast to TD antigens, TI antigens do not induce classical antibody isotype switch and high affinity memory B cells (54). Yet, purified CPSs from *S. pneumoniae* (Pneumovax® - 23 valent) and from Group B *Streptococcus* (GBS) serotype III can induce not only IgM but also IgG antibody responses in mice and in adults without adjuvant (19, 55-58). On the contrary, *S. suis* serotype 2 CPS alone is unable to induce any significant antibody response, even when adjuvanted with TiterMax Gold or STIMUNE® or when combined with the TLR-ligand CpG in mice (unpublished results). Furthermore, previous studies using live *S. suis* serotype 2 infection showed modest IgM and no isotype-switched IgG anti-CPS antibody titers in pigs and in mice even after an experimental re-infection (17). Thus, *S. suis* serotype 2 CPS seems to be particularly non-immunogenic, making it an ideal candidate for evaluation of the effect of glycoconjugation on immunogenicity. A precedent exists in the literature where serotype 2 CPS was conjugated to bovine serum albumin with the aim to obtain anti-CPS control sera for *in vitro* studies (59). Yet, neither the biochemical characteristics nor the immunogenicity and functional activity of that conjugate were investigated.

To produce the glycoconjugate, it was first decided to depolymerize the CPS to a smaller size in order to improve the efficacy of the conjugation and the residual exposure of the T cell peptide epitopes of the protein carrier. Ultrasonic irradiation (sonication) was chosen over fragmentation by chemical (60-62) or enzymatic (63-66) methods to avoid chemical alterations (67). In addition, it is easy to use, reliable for labile epitopes and does not require elimination of excess reagents. Another great advantage of ultrasonic irradiation is the reduction in sample polydispersity (39), facilitating biochemical characterization, particularly within the glycoconjugate.

In order to conjugate the CPS to a protein carrier by reductive amination, aldehydes must be introduced, ideally regioselectively, in the CPS by oxidation with sodium periodate. The presence of sialic acid offers a unique opportunity to use mild oxidative conditions, leaving the remainder of the polysaccharide unmodified, as already described for conjugates against different serotypes of GBS (41, 68-73). The glycerol side chain of the sialic acid residues is particularly reactive toward mild periodate oxidation. The desired percentage of oxidation is also a critical parameter: too little reactive groups will yield a poor conjugate while too many will leave few intact epitopes of the native polysaccharide (40). For *S. suis* type 2, we found that a low oxidation level (10%) yielded immunogenic conjugates and was thus preferred over very high oxidation levels. In accordance, some immunogenic conjugates for GBS types Ia, Ib, and II have been described with an oxidation level of 10% (68-70). In contrast, conjugates of other GBS types, including type III, routinely use higher oxidation levels of 25% (41, 70, 71, 73) or between 40-50% (72), and could go up to 66% without loss of immunogenicity for the serotype III (74), even though the type III GBS CPS conformational epitope is controlled by the sialic acid residue (75, 76).

Two conjugate vaccine formulations were obtained with different CPS:TT ratios. The 2:1 mixed conjugate was found to be the most immunogenic, namely resulting in significantly higher titers of IgG2b and IgG2c anti-CPS isotypes. This difference in immunogenicity may arise from the higher percentage of total CPS in the 2:1 than in the 1:1 mixed conjugate, which might influence the capacity of the conjugate to modulate the immune cells, including antigen-presenting cells (APCs), presumably through its higher molecular mass/size that might ease

uptake and internalization. In this regard, it has been shown that the polysaccharide size used for conjugation, the obtained conjugate size, and the degree of polysaccharide-protein cross-linking influence the immunogenicity and protective efficacy of a GBS type III-TT conjugate vaccine (74, 77, 78). Further studies evaluating how the aforementioned parameters affect the immunogenicity of a *S. suis* type 2 CPS conjugate, which can contribute to improvements of the design of the conjugate vaccine, are underway.

During pre-trials, it was observed that conjugation alone was not enough to induce a robust immunological response against *S. suis* type 2 CPS (data not shown). In this regard, subunit vaccines are known to induce more potent and durable antigen-specific immunity if combined with an adjuvant (79). It has been shown that adjuvants can not only improve the immunogenicity of conjugate vaccines, but they can also direct the anti-polysaccharide antibody isotype switch towards the desired IgG subclasses (80, 81). CpG ODN 1826 (82), was shown to enhance the isotype switching from IgM to IgG2a and IgG3 subclasses for pneumococcal conjugates in a serotype- and mouse age-dependent manner (43, 80, 83). In our study, CpG ODN 1826 failed to significantly improve the immunogenicity of the *S. suis* type 2 CPS mixed conjugate or to induce isotype switching. Conditions differing from those used in this study, such as the vaccine and adjuvant doses, might explain the observed differences. The biochemical properties of different CPS might also influence the adjuvant capacity of CpG (83). As such, the choice of adjuvant is vital to maximize isotype switching. In the present study, high levels of IgM as well as of both Th1 (IgG2b, IgG2c, and IgG3) and Th2 (IgG1) IgG subclasses were observed in mice immunized with conjugates in TiterMax Gold®. Similarly, STIMUNE® induced high levels of IgM and a mixed Th1/Th2 IgG response. However, the Th1 antibody response was restricted to the IgG2b subclass when using STIMUNE®. Our data is in agreement with previous studies suggesting that water-in-oil adjuvants, like TiterMax Gold® and STIMUNE®, induce strong humoral responses by means of the depot effect and coimmunostimulatory properties, which are associated with mixed Th1 and Th2 antibody responses (46, 84-86). While STIMUNE® is principally composed of purified and defined mineral oil, TiterMax Gold® includes metabolizable squalene as the oil-phase and also includes block copolymers in the formulation. This special formulation might contribute to the increased isotype switching activity observed with this adjuvant in mice.

These block copolymers have been shown to influence the localization and retention of antigen in lymphoid tissues and to recruit and activate APCs and lymphocytes (48, 87).

However, even when using TiterMax Gold as adjuvant, not all mice developed anti-CPS IgG subclass-switched antibodies, although they all exhibited strong anti-CPS IgM titers in addition to high antibody levels to TT. Isotype switching from IgM to IgG depends on the signals (cytokines and co-stimulatory molecules) provided by APCs to T cells, and then by T cells to B cells. These signals are induced by TT and the adjuvant since the CPS is not immuno-stimulatory *per se* (24, 48). The intensity of these signals might vary among individual mice. As such, some of them will not be able to switch or will present different isotype profiles compared to other individuals from the same group.

When compared to the conjugate vaccine, a TiterMax Gold-adjuvanted control mixture of free CPS and TT induced a reduced TD anti-CPS antibody response. This also confirms that the immunogenicity in the 2:1 mixed conjugate is in fact due to the conjugate present in the vaccine formulation. Free polysaccharides in conjugate vaccines have been shown to have adverse effects on the immune response (by presenting mixed TI and TD forms of the CPS antigen) (88), as demonstrated with pneumococcal CPS types 3, 4, 6B, and 14 (89-91). In contrast, based on our results, free *S. suis* CPS does not seem to influence the immunogenicity of the conjugate vaccine. This is suggested by the fact that no suppressive effect was observed when using high doses of vaccine preparations and that the 2:1 fractionated conjugate behaves similarly to the mixed vaccine conjugate. The 2:1 mixed conjugate vaccine was also sufficient to induce protection in pigs, rendering the purification of the post-conjugation preparation by chromatography unnecessary for potential use in the field.

While ELISA titers are great tools to evaluate the immunogenicity of vaccine prototypes, they are not generally considered reliable correlates of immunity. A strong antibody response does not necessarily reflect upon the protection of an individual (38). In this regard, functional assays are preferred, like the OPA, a recognized correlate of protective immunity against extracellular encapsulated Gram-positive bacteria (18, 92). During the OPA, opsonizing antibodies from the immunized serum will opsonize the target bacteria, which

results in bacterial phagocytosis and bactericidal activity (38, 93-95). Specific cell type activation depends on the Ig isotypes/subclasses present in the immune serum, since each isotype/subclass possesses different binding preferences to Fc receptors, which differently influences the cell response (38, 94). Besides IgM, the predominant subclass of protective antibodies to TI antigens in mice is the IgG3 (54, 96-98). A study using mouse monoclonal antibodies proposed that the type 1 subclasses (IgG3 >> IgG2b ≥ IgG2a) are superior in both opsonophagocytosis activity and complement activation than the type 2 IgG1subclass. Yet, these functional properties of mouse IgG subclasses seem to depend on the target antigen (protein *vs.* carbohydrate), antigen distribution, and the susceptibility of the bacteria for antibody/complement attack (99, 100). In our OPA experiments, the two groups which obtained the highest bacterial killing values were the 2:1 mixed or fractionated conjugate adjuvanted with TiterMax Gold®, both containing the highest titers of all type 1 IgG subclasses. They were closely followed by the 2:1 mixed conjugate adjuvanted with STIMUNE®, although this group lacks production of IgG3 and IgG2c. It has been shown that mice lacking the dominant IgG3 subclass made to bacterial CPS are more susceptible to fatal *S. pneumoniae* sepsis than wild-type mice, yet, these mice can be rescued by induction of IgG1 using a *S. pneumoniae* glycoconjugate (100). In the $IgG3^{-/-}$ mouse model, it was proposed that low titers of IgG1 anti-CPS antibodies in combination with complement deposition mediated by IgM anti-CPS antibodies may have been adequate to opsonize pneumococci for uptake by macrophages (100). Thus, in the absence of high levels of the most opsonizing antibodies (such as IgG3), other Ig isotypes/subclasses might compensate. As such, protection depends on the right balance between functionality, affinity and quantity of different anti-CPS Ig isotypes/subclasses induced by the vaccine. In this regard, control mouse groups immunized with the mixture of free CPS and free TT or mice hyperimmunized with killed-bacteria failed to adequately perform in the OPA test, probably due to the combined absence or low levels of several IgG subclasses, including IgG1. Together with the absence of protection in placebo groups, the OPA data confirm that anti-CPS antibodies generated by the glycoconjugate play a crucial role in opsonophagocytosis of *S. suis*, and thus in the protective effect observed with the vaccine.

For an ultimate proof of concept, evaluation of the conjugate was conducted in *S. suis'* natural host: the pig. In the field, pigs usually present clinical signs between 5 – 10 weeks of age (3), which would correspond approximately to 7 – 14 days post-boost. Pigs immunized with the 2:1 mixed conjugate showed significant protection against a challenge systemic infection. As aforementioned, the combined action of IgM and IgG1 might be sufficient to confer protection in the swine model, yet the functional activity of different swine isotypes/subclasses remains to be elucidated. During swine immunization trials, a bacterin adjuvanted with STIMUNE® was used as control. Although the bacterin induced similar levels of protection than the conjugate vaccine, this protection was not related to anti-CPS antibodies but probably related to anti-protein antibodies. Yet, and in contrast to CPS, which is a universal antigen for *S. suis* type 2, protein antigens vary upon the strain origin or sequence type (ST) within the same serotype (16, 101-105). Strains belonging to the same ST (ST1) were used as bacterin and challenge strains, thus the capacity of such a vaccine preparation to protect against a heterologous challenge (ST25 and ST28 strains) remains to be elucidated (2). It can, however, be expected that protection conferred by a bacterin might be strain/ST-dependent, while that provided by a CPS conjugate vaccine is serotype-specific and thus strain/ST-independent (106).

In conclusion, conception and laboratory-scale production of a glycoconjugate vaccine against *S. suis* serotype 2 is reported for the first time. In its unpurified form as desalted post-conjugation formulation (mixed conjugate), the vaccine was shown to be efficient when emulsified with water-in-oil adjuvants. In mice, anti-CPS IgM and isotype-switched IgG antibodies were observed and found to be functional in an *in vitro* opsonophagocytosis assay. In pigs, anti-CPS IgM and IgG1 antibodies were detected and found to be significantly protective in an *in vivo* lethal-dose challenge with virulent *S. suis* serotype 2. At this stage, these results represent a proof of concept for the potential of glycoconjugate vaccines in veterinary medicine applications against invasive bacterial infections.

References

1. Gottschalk M. Streptococcosis. In: Zimmerman JJ, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of swine. 10th ed. Ames, USA: Wiley-Blackwell Publishing; 2012. p. 841-55.
2. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect. 2014;3(6):e45.
3. Higgins R, Gottschalk M. Streptococcal diseases. In: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ, editors. Diseases of swine. 9th ed. Ames, USA: Blackwell Publishing; 2006. p. 769-83.
4. Sanford SE, Tilker ME. *Streptococcus suis* type II-associated diseases in swine: observations of a one-year study. J Am Vet Med Assoc. 1982;181(7):673-6.
5. Cloutier G, D'Allaire S, Martinez G, Surprenant C, Lacouture S, Gottschalk M. Epidemiology of *Streptococcus suis* serotype 5 infection in a pig herd with and without clinical disease. Vet Microbiol. 2003;97(1–2):135-51.
6. Segura M. Fisher scientific award lecture - The capsular polysaccharides of Group B *Streptococcus* and *Streptococcus suis* differently modulate bacterial interactions with dendritic cells. Can J Microbiol. 2012;58(3):249-60.
7. Holt ME, Enright MR, Alexander TJ. Immunisation of pigs with live cultures of *Streptococcus suis* type 2. Res Vet Sci. 1988;45(3):349-52.
8. Holt ME, Enright MR, Alexander TJ. Studies of the protective effect of different fractions of sera from pigs immune to *Streptococcus suis* type 2 infection. J Comp Pathol. 1989;100(4):435-42.
9. Blouin C, Higgins R, Gottschalk M, Simard J. Evaluation of the antibody response in pigs vaccinated against *Streptococcus suis* capsular type 2 using a double-antibody sandwich enzyme-linked immunosorbent assay. Can J Vet Res. 1994;58(1):49-54.
10. Lapointe L, D'Allaire S, Lebrun A, Lacouture S, Gottschalk M. Antibody response to an autogenous vaccine and serologic profile for *Streptococcus suis* capsular type 1/2. Can J Vet Res. 2002;66(1):8-14.
11. Baums CG, Brüggemann C, Kock C, Beineke A, Waldmann K-H, Valentin-Weigand P. Immunogenicity of an autogenous *Streptococcus suis* bacterin in preparturient sows and their piglets in relation to protection after weaning. Clin Vaccine Immunol. 2010;17(10):1589-97.
12. Wisselink HJ, Stockhofe-Zurwieden N, Hilgers LAT, Smith HE. Assessment of protective efficacy of live and killed vaccines based on a non-encapsulated mutant of *Streptococcus suis* serotype 2. Vet Microbiol. 2002;84(1–2):155-68.
13. Segura M. *Streptococcus suis* vaccines: candidate antigens and progress. Expert Rev Vaccines. 2015;14(12):1587-608.
14. Busque P, Higgins R, Caya F, Quessy S. Immunization of pigs against *Streptococcus suis* serotype 2 infection using a live avirulent strain. Can J Vet Res. 1997;61(4):275-9.
15. Fittipaldi N, Harel J, D'Amours B, Lacouture S, Kobisch M, Gottschalk M. Potential use of an unencapsulated and aromatic amino acid-auxotrophic *Streptococcus suis* mutant as a live attenuated vaccine in swine. Vaccine. 2007;25(18):3524-35.

16. Fittipaldi N, Segura M, Grenier D, Gottschalk M. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent *Streptococcus suis*. Future Microbiol. 2012;7(2):259-79.
17. Calzas C, Lemire P, Auray G, Gerdts V, Gottschalk M, Segura M. Antibody response specific to the capsular polysaccharide is impaired in *Streptococcus suis* serotype 2-infected animals. Infect Immun. 2015;83(1):441-53.
18. Song JY, Moseley MA, Burton RL, Nahm MH. Pneumococcal vaccine and opsonic pneumococcal antibody. J Infect Chemother. 2013;19(3):412-25.
19. Heath PT. An update on vaccination against Group B *Streptococcus*. Expert Rev Vaccines. 2011;10(5):685-94.
20. Bottomley MJ, Serruto D, Sáfadi MAP, Klugman KP. Future challenges in the elimination of bacterial meningitis. Vaccine. 2012;30(Supplement 2):B78-B86.
21. Roy R, Shiao TC. Organic chemistry and immunochemical strategies in the design of potent carbohydrate-based vaccines. Chimia (Aarau). 2011;65(1-2):24-9.
22. Elliott SD, Clifton-Hadley F, Tai J. Streptococcal infection in young pigs. V. An immunogenic polysaccharide from *Streptococcus suis* type 2 with particular reference to vaccination against streptococcal meningitis in pigs. J Hyg (Lond). 1980;85(2):275-85.
23. Charland N, Jacques M, Lacouture S, Gottschalk M. Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by *Streptococcus suis* serotypes 1, 2 and 1/2. Microbiology. 1997;143(11):3607-14.
24. Knuf M, Kowalzik F, Kieninger D. Comparative effects of carrier proteins on vaccine-induced immune response. Vaccine. 2011;29(31):4881-90.
25. Byrd W, Kadis S. Preparation, characterization, and immunogenicity of conjugate vaccines directed against *Actinobacillus pleuropneumoniae* virulence determinants. Infect Immun. 1992;60(8):3042-51.
26. Byrd W, Harmon BG, Kadis S. Protective efficacy of conjugate vaccines against experimental challenge with porcine *Actinobacillus pleuropneumoniae*. Vet Immunol Immunopathol. 1992;34(3-4):307-24.
27. Andresen LO, Jacobsen MJ, Nielsen JP. Experimental vaccination of pigs with an *Actinobacillus pleuropneumoniae* serotype 5b capsular polysaccharide-tetanus toxoid conjugate. Acta Vet Scand. 1997;38(3):283-93.
28. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. Biochem Cell Biol. 2010;88(3):513-25.
29. Segura M, Stankova J, Gottschalk M. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect Immun. 1999;67(9):4646-54.
30. Calzas C, Goyette-Desjardins G, Lemire P, Gagnon F, Lachance C, Van Calsteren MR, et al. Group B *Streptococcus* and *Streptococcus suis* capsular polysaccharides induce chemokine production by dendritic cells via Toll-like receptor 2- and MyD88-dependent and -independent pathways. Infect Immun. 2013;81(9):3106-18.
31. Van Calsteren MR, Gagnon F, Calzas C, Goyette-Desjardins G, Okura M, Takamatsu D, et al. Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide. Biochem Cell Biol. 2013;91(2):49-58.
32. Houde M, Gottschalk M, Gagnon F, Van Calsteren M-R, Segura M. *Streptococcus suis* Capsular Polysaccharide Inhibits Phagocytosis through Destabilization of Lipid

Microdomains and Prevents Lactosylceramide-Dependent Recognition. Infect Immun. 2012;80(2):506-17.
33. Lecours MP, Fittipaldi N, Takamatsu D, Okura M, Segura M, Goyette-Desjardins G, et al. Sialylation of *Streptococcus suis* serotype 2 is essential for capsule expression but is not responsible for the main capsular epitope. Microbes Infect. 2012;14(11):941-50.
34. Lachance C, Gottschalk M, Gerber PP, Lemire P, Xu J, Segura M. Exacerbated type II interferon response drives hypervirulence and toxic shock by an emergent epidemic strain of *Streptococcus suis*. Infect Immun. 2013;81(6):1928-39.
35. Lachance C, Segura M, Gerber PP, Xu J, Gottschalk M. Toll-like receptor 2-independent host innate immune response against an epidemic strain of *Streptococcus suis* that causes a toxic shock-like syndrome in humans. PLoS One. 2013;8(5):e65031.
36. U.S. Code of Federal Regulations. Title 9-Animals and Animal Products, Chapter I-Animal and Plant Health Inspection Service, Department of Agriculture; Subchapter E; Part 117.4 Test animals. 1998.
37. Higgins R, Gottschalk M. An update on *Streptococcus suis* identification. J Vet Diagn Invest. 1990;2(3):249-52.
38. Goyette-Desjardins G, Roy R, Segura M. Murine whole-blood opsonophagocytosis assay to evaluate protection by antibodies raised against encapsulated extracellular bacteria. In: Lepenies B, editor. Carbohydrate-Based Vaccines - Methods and Protocols. Methods in Molecular Biology. 1331. New York, USA: Springer; 2015. p. 81-92.
39. Szu SC, Zon G, Schneerson R, Robbins JB. Ultrasonic irradiation of bacterial polysaccharides. Characterization of the depolymerized products and some applications of the process. Carbohydr Res. 1986;152(0):7-20.
40. Reuter G, Schauer R, Szeiki C, Kamerling JP, Vliegenthart JF. A detailed study of the periodate oxidation of sialic acids in glycoproteins. Glycoconj J. 1989;6(1):35-44.
41. Wessels MR, Paoletti LC, Kasper DL, DiFabio JL, Michon F, Holme K, et al. Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against type III Group B *Streptococcus*. J Clin Invest. 1990;86(5):1428-33.
42. Merril C, Goldman D, Sedman S, Ebert M. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science. 1981;211(4489):1437-8.
43. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J Exp Med. 1997;186(10):1623-31.
44. Stills HF. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR Journal. 2005;46(3):280-93.
45. Leenaars PPAM, Hendriksen CFM, Angulo AF, Koedam MA, Claassen E. Evaluation of several adjuvants as alternatives to the use of Freund's adjuvant in rabbits. Vet Immunol Immunopathol. 1994;40(3):225-41.
46. Bennett B, Check IJ, Olsen MR, Hunter RL. A comparison of commercially available adjuvants for use in research. J Immunol Methods. 1992;153(1–2):31-40.
47. Sommariva M, de Cesare M, Meini A, Cataldo A, Zaffaroni N, Tagliabue E, et al. High efficacy of CpG-ODN, cetuximab and cisplatin combination for very advanced ovarian xenograft tumors. J Transl Med. 2013;11:25.
48. Jennings VM. Review of selected adjuvants used in antibody production. ILAR Journal. 1995;37(3):119-25.

49. Kateregga J, Lubega GW, Lindblad EB, Authie E, Coetzer TH, Boulange AF. Effect of adjuvants on the humoral immune response to congopain in mice and cattle. BMC Vet Res. 2012;8:63.
50. Dalsgaard K, Hilgers L, Trouve G. Classical and new approaches to adjuvant use in domestic food animals. Adv Vet Sci Comp Med. 1990;35:121-60.
51. Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol. 2010;17(7):1055-65.
52. Swildens B, Nielen M, Wisselink HJ, Verheijden JHM, Stegeman JA. Elimination of strains of *Streptococcus suis* serotype 2 from the tonsils of carrier sows by combined medication and vaccination. Vet Rec. 2007;160(18):619-21.
53. Gilbert FB, Poutrel B, Sutra L. Immunogenicity in cows of *Staphylococcus aureus* type 5 capsular polysaccharide—ovalbumin conjugate. Vaccine. 1994;12(4):369-74.
54. Lee C-J, Lee LH, Frasch CE. Protective immunity of pneumococcal glycoconjugates. Crit Rev Microbiol. 2003;29(4):333-49.
55. Baker CJ, Rench MA, Edwards MS, Carpenter RJ, Hays BM, Kasper DL. Immunization of pregnant women with a polysaccharide vaccine of Group B *Streptococcus*. N Engl J Med. 1988;319(18):1180-5.
56. Kasper DL, Paoletti LC, Wessels MR, Guttormsen HK, Carey VJ, Jennings HJ, et al. Immune response to type III Group B streptococcal polysaccharide-tetanus toxoid conjugate vaccine. J Clin Invest. 1996;98(10):2308-14.
57. Moens L, Jeurissen A, Nierkens S, Boon L, Van Kaer L, Kasran A, et al. Generation of antibody responses to pneumococcal capsular polysaccharides is independent of CD1 expression in mice. Infect Immun. 2009;77(5):1976-80.
58. Schütz K, Hughes R, Parker A, Quinti I, Thon V, Cavaliere M, et al. Kinetics of IgM and IgA antibody response to 23-valent pneumococcal polysaccharide vaccination in healthy subjects. J Clin Immunol. 2013;33(1):288-96.
59. Baums CG, Kock C, Beineke A, Bennecke K, Goethe R, Schröder C, et al. *Streptococcus suis* bacterin and subunit vaccine immunogenicities and protective efficacies against serotypes 2 and 9. Clin Vaccine Immunol. 2009;16(2):200-8.
60. Duan J, Kasper DL. Oxidative depolymerization of polysaccharides by reactive oxygen/nitrogen species. Glycobiology. 2011;21(4):401-9.
61. Higashi K, Ly M, Wang Z, Masuko S, Bhaskar U, Sterner E, et al. Controlled photochemical depolymerization of K5 heparosan, a bioengineered heparin precursor. Carbohydr Polym. 2011;86(3):1365-70.
62. Anderson P. Antibody responses to *Haemophilus influenzae* type b and diphtheria toxin induced by conjugates of oligosaccharides of the type b capsule with the nontoxic protein CRM197. Infect Immun. 1983;39(1):233-8.
63. Svenson SB, Lindberg AA. Oligosaccharide-protein conjugate: A novel approach for making *Salmonella* O-antigen immunogens. FEMS Microbiol Lett. 1977;1(3):145-7.
64. Svenson SB, Lindberg AA. Coupling of acid labile *Salmonella* specific oligosaccharides to macromolecular carriers. J Immunol Methods. 1979;25(4):323-35.
65. Wessels MR, Muñoz A, Kasper DL. A model of high-affinity antibody binding to type III Group B *Streptococcus* capsular polysaccharide. Proc Natl Acad Sci U S A. 1987;84(24):9170-4.

66. Paoletti LC, Kasper DL, Michon F, DiFabio J, Holme K, Jennings HJ, et al. An oligosaccharide-tetanus toxoid conjugate vaccine against type III Group B *Streptococcus*. J Biol Chem. 1990;265(30):18278-83.
67. Pawlowski A, Svenson SB. Electron beam fragmentation of bacterial polysaccharides as a method of producing oligosaccharides for the preparation of conjugate vaccines. FEMS Microbiol Lett. 1999;174(2):255-63.
68. Paoletti LC, Wessels MR, Michon F, DiFabio J, Jennings HJ, Kasper DL. Group B *Streptococcus* type II polysaccharide-tetanus toxoid conjugate vaccine. Infect Immun. 1992;60(10):4009-14.
69. Wessels MR, Paoletti LC, Rodewald AK, Michon F, DiFabio J, Jennings HJ, et al. Stimulation of protective antibodies against type Ia and Ib Group B *Streptococci* by a type Ia polysaccharide-tetanus toxoid conjugate vaccine. Infect Immun. 1993;61(11):4760-6.
70. Paoletti LC, Wessels MR, Rodewald AK, Shroff AA, Jennings HJ, Kasper DL. Neonatal mouse protection against infection with multiple Group B streptococcal (GBS) serotypes by maternal immunization with a tetravalent GBS polysaccharide-tetanus toxoid conjugate vaccine. Infect Immun. 1994;62(8):3236-43.
71. Wessels MR, Paoletti LC, Pinel J, Kasper DL. Immunogenicity and protective activity in animals of a type V Group B streptococcal polysaccharide-tetanus toxoid conjugate vaccine. J Infect Dis. 1995;171(4):879-84.
72. Paoletti LC, Pinel J, Johnson KD, Reinap B, Ross RA, Kasper DL. Synthesis and preclinical evaluation of glycoconjugate vaccines against Group B *Streptococcus* types VI and VIII. J Infect Dis. 1999;180(3):892-5.
73. Paoletti LC, Kasper DL. Conjugate vaccines against Group B *Streptococcus* types IV and VII. J Infect Dis. 2002;186(1):123-6.
74. Wessels MR, Paoletti LC, Guttormsen HK, Michon F, D'Ambra AJ, Kasper DL. Structural properties of Group B streptococcal type III polysaccharide conjugate vaccines that influence immunogenicity and efficacy. Infect Immun. 1998;66(5):2186-92.
75. Jennings HJ, Lugowski C, Kasper DL. Conformational aspects critical to the immunospecificity of the type III Group B streptococcal polysaccharide. Biochemistry. 1981;20(16):4511-8.
76. Zou W, Mackenzie R, Thérien L, Hirama T, Yang Q, Gidney MA, et al. Conformational epitope of the type III Group B *Streptococcus* capsular polysaccharide. J Immunol. 1999;163(2):820-5.
77. Avci FY, Li X, Tsuji M, Kasper DL. A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. Nat Med. 2011;17(12):1602-9.
78. Michon F, Uitz C, Sarkar A, D'Ambra AJ, Laude-Sharp M, Moore S, et al. Group B streptococcal type II and III conjugate vaccines: physicochemical properties that influence immunogenicity. Clin Vaccine Immunol. 2006;13(8):936-43.
79. O'Hagan DT, Valiante NM. Recent advances in the discovery and delivery of vaccine adjuvants. Nat Rev Drug Discov. 2003;2(9):727-35.
80. Chu RS, McCool T, Greenspan NS, Schreiber JR, Harding CV. CpG oligodeoxynucleotides act as adjuvants for pneumococcal polysaccharide-protein

conjugate vaccines and enhance antipolysaccharide immunoglobulin G2a (IgG2a) and IgG3 antibodies. Infect Immun. 2000;68(3):1450-6.
81. Fattom A, Li X, Cho YH, Burns A, Hawwari A, Shepherd SE, et al. Effect of conjugation methodology, carrier protein, and adjuvants on the immune response to *Staphylococcus aureus* capsular polysaccharides. Vaccine. 1995;13(14):1288-93.
82. Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM. CpG DNA as a vaccine adjuvant. Expert Rev Vaccines. 2011;10(4):499-511.
83. Kovarik J, Bozzotti P, Tougne C, Davis HL, Lambert PH, Krieg AM, et al. Adjuvant effects of CpG oligodeoxynucleotides on responses against T-independent type 2 antigens. Immunology. 2001;102(1):67-76.
84. Cox JC, Coulter AR. Adjuvants—A classification and review of their modes of action. Vaccine. 1997;15(3):248-56.
85. Leenaars PP, Savelkoul HF, Hendriksen CF, Van Rooijen N, Claassen E. Increased adjuvant efficacy in stimulation of antibody responses after macrophage elimination in vivo. Immunology. 1997;90(3):337-43.
86. Heegaard P, Dedieu L, Johnson N, Le Potier M-F, Mockey M, Mutinelli F, et al. Adjuvants and delivery systems in veterinary vaccinology: current state and future developments. Arch Virol. 2011;156(2):183-202.
87. Hunter RL. Overview of vaccine adjuvants: present and future. Vaccine. 2002;20 Suppl 3:S7-12.
88. Peeters CC, Lagerman PR, Weers O, Oomen LA, Hoogerhout P, Beurret M, et al. Preparation of polysaccharide-conjugate vaccines. In: Robinson A, Hudson MJ, Cranage MP, editors. Methods in Molecular Medecine - Vaccine Protocols. 87. 2nd ed. Totowa, USA: Humana Press Inc; 2003. p. 153-73.
89. Wilson D, Braley-Mullen H. Antigen requirements for priming of type III pneumococcal polysaccharide-specific IgG memory responses: suppression of memory with the T-independent form of antigen. Cell Immunol. 1981;64(1):177-86.
90. Peeters CCAM, Tenbergen-Meekes A-MJ, Poolman JT, Zegers BJM, Rijkers GT. Immunogenicity of a *Streptococcus pneumoniae* type 4 polysaccharide-protein conjugate vaccine is decreased by admixture of high doses of free saccharide. Vaccine. 1992;10(12):833-40.
91. Rodriguez ME, van den Dobbelsteen GP, Oomen LA, de Weers O, van Buren L, Beurret M, et al. Immunogenicity of *Streptococcus pneumoniae* type 6B and 14 polysaccharide-tetanus toxoid conjugates and the effect of uncoupled polysaccharide on the antigen-specific immune response. Vaccine. 1998;16(20):1941-9.
92. Romero-Steiner S, Frasch CE, Carlone G, Fleck RA, Goldblatt D, Nahm MH. Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. Clin Vaccine Immunol. 2006;13(2):165-9.
93. Guilliams M, Bruhns P, Saeys Y, Hammad H, Lambrecht BN. The function of Fc receptors in dendritic cells and macrophages. Nat Rev Immunol. 2014;14(2):94-108.
94. Underhill DM, Ozinsky A. Phagocytosis of microbes: complexity in action. Annu Rev Immunol. 2002;20(1):825-52.
95. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol. 2010;11(9):785-97.
96. Perlmutter RM, Hansburg D, Briles DE, Nicolotti RA, David JM. Subclass restriction of murine anti-carbohydrate antibodies. J Immunol. 1978;121(2):566-72.

97. Rubinstein LJ, Stein KE. Murine immune response to the *Neisseria meningitidis* group C capsular polysaccharide. I. Ontogeny. J Immunol. 1988;141(12):4352-6.
98. Schreiber JR, Cooper LJN, Diehn S, Dahlhauser PA, Tosi MF, Glass DD, et al. Variable region-identical monoclonal antibodies of different IgG subclass directed to *Pseudomonas aeruginosa* lipopolysaccharide O-specific side chain function differently. J Infect Dis. 1993;167(1):221-6.
99. Michaelsen TE, Kolberg J, Aase A, Herstad TK, Høiby EA. The four mouse IgG isotypes differ extensively in bactericidal and opsonophagocytic activity when reacting with the P1.16 epitope on the outer membrane PorA protein of *Neisseria meningitidis*. Scand J Immunol. 2004;59(1):34-9.
100. McLay J, Leonard E, Petersen S, Shapiro D, Greenspan NS, Schreiber JR. γ3 gene-disrupted mice selectively deficient in the dominant IgG subclass made to bacterial polysaccharides. II. Increased susceptibility to fatal pneumococcal sepsis due to absence of anti-polysaccharide IgG3 is corrected by induction of anti-polysaccharide IgG1. J Immunol. 2002;168(7):3437-43.
101. Galina L, Vecht U, Wisselink HJ, Pijoan C. Prevalence of various phenotypes of *Streptococcus suis* isolated from swine in the U.S.A. based on the presence of muraminidase-released protein and extracellular factor. Can J Vet Res. 1996;60(1):72-4.
102. Gottschalk M, Lebrun A, Wisselink H, Dubreuil JD, Smith H, Vecht U. Production of virulence-related proteins by Canadian strains of *Streptococcus suis* capsular type 2. Can J Vet Res. 1998;62(1):75-9.
103. Okwumabua O, Abdelmagid O, Chengappa MM. Hybridization analysis of the gene encoding a hemolysin (suilysin) of *Streptococcus suis* type 2: evidence for the absence of the gene in some isolates. FEMS Microbiol Lett. 1999;181(1):113-21.
104. Fittipaldi N, Xu J, Lacouture S, Tharavichitkul P, Osaki M, Sekizaki T, et al. Lineage and virulence of *Streptococcus suis* serotype 2 isolates from North America. Emerg Infect Dis. 2011;17(12):2239-44.
105. Li Y, Martinez G, Gottschalk M, Lacouture S, Willson P, Dubreuil JD, et al. Identification of a surface protein of *Streptococcus suis* and evaluation of its immunogenic and protective capacity in pigs. Infect Immun. 2006;74(1):305-12.
106. Van Calsteren MR, Goyette-Desjardins G, Gagnon F, Okura M, Takamatsu D, Roy R, et al. Explaining the serological characteristics of *Streptococcus suis* serotypes 1 and 1/2 from their capsular polysaccharide structure and biosynthesis. J Biol Chem. 2016;291(16):8387-98.

Tables

Table 1. Clinical evaluation of immunized pigs after experimental challenge with *S. suis* serotype 2[1].

	Abnormal behavior		Abnormal locomotion		CNS clinical signs	
Groups	%[2]	*P* value[3]	%[2]	*P* value[3]	%[2]	*P* value[3]
Type 2 Bacterin	31.6	0.0209	26.3	0.0064	1.2	NS
2:1 Mixed Conjugate	28.1	0.0308	33.5	0.0335	0	NS
Placebo (challenge control)	90.7	-	89.3	-	2.6	-

[1] Assessed were behavior, including any behavior indicating an effect of challenge on the central nervous system (CNS) and locomotion. The observations for behavior were numerically scored as follows: 0 = physiological, 1 = depression, 2 = apathy. Observations for locomotion were scored as 0 = physiological, 1 = slightly to moderately lame, 2 = severely lame/ reluctant to stand, 3 = animal partially /completely down; animals can rise, but lie down again within 10 seconds. CNS signs were scored as 0 = absent and 1 = present.

[2] Assessment of cumulative observation period. Percent of evaluations where behavior, locomotion or CNS signs gave a value > 0 across days. Data are expressed as least squares means (back-transformed, %).

[3] Adjusted *P*-value (*Scheffé*'s test): all values compared to challenge control group; strict control group not included in assessment. NS, non-significant with $P \geq 0.05$.

Table S1. Size-exclusion chromatography coupled with multi-angle light scattering (SEC–MALS) data for the depolymerized polysaccharide lots.

Depolymerized CPS[1]	M_w (g/mol)	R_z (nm)	M_w/M_n
Lot I	1.128×10^5 (0.02%)	14.0 (0.2%)	1.003 (0.02%)
Lot II	1.180×10^5 (0.05%)	17.6 (0.3%)	1.001 (0.07%)

[1] Depolymerized polysaccharide was obtained by ultrasonic irradiation.

Note: M_w, weight-average molar mass; R_z, z-average radius of gyration; M_w/M_n, polydispersity. Values in parentheses represent relative standard deviations.

Table S2. Gross pathology observations from necropsy (or post-mortem examination) of challenged pigs[1].

	Thoracic Cavity		Joint	
Groups	%[2]	*P* value[3]	%[2]	*P* value[3]
Type 2 Bacterin	35.7	0.0294	35.7	NS
2:1 Mixed Conjugate	40.0	NS	30.0	NS
Placebo (challenge control)	80.0	-	53.3	-

[1] Signs of inflammation of the thoracic cavity (including serosal surfaces, heart and lung) and the joints (including excess fluid, fibrin, swelling) were recorded.

[2] Percentage of animals with pathological findings/observations.

[3] *P*-value: all values compared to challenge control group; strict control group not included in assessment. NS, non-significant with $P \geq 0.05$.

Table S3. *Streptococcus suis* serotype 2 recovery from swabs at necropsy (or post-mortem examination) of challenged pigs[1].

	Meninges		Pericard		Joints	
Groups	%[2]	*P* value[3]	%[2]	*P* value[3]	%[2]	*P* value[3]
Type 2 Bacterin	21.4	0.0092	14.3	0.0078	14.3	0.0007
2:1 Mixed Conjugate	40.0	NS	30.0	NS	20.0	0.0051
Placebo (challenge control)	80.0	-	66.7	-	80.0	-

[1] Culture from swabs were confirmed by morphology, serotyping with type 2 antisera and by *S. suis* type 2 PCR.

[2] Percentage of animals with at least one positive *S. suis* type 2 isolate from swab cultures.

[3] *P*-value : all values compared to challenge control group; strict control group not included in assessment. NS, non-significant with $P \geq 0.05$.

Figures

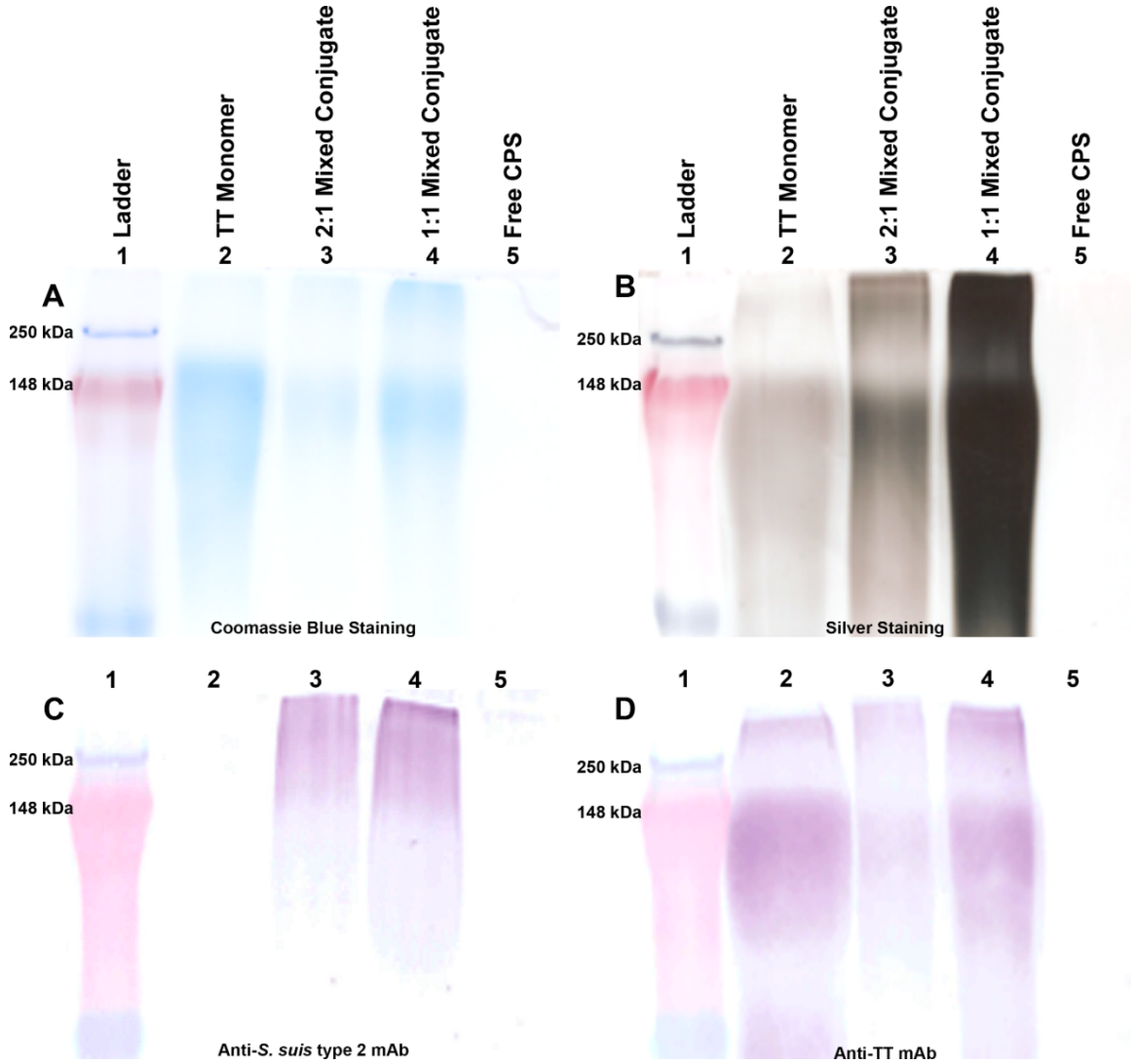


Figure 1. SDS-PAGE and Western blot characterization of the glycoconjugates.

Separating gels containing 7.5% acrylamide were either stained by Coomassie Blue Stain (A) or Silver Stain (B), or transferred onto a nitrocellulose membrane and revealed by immunoblotting with either an anti-*S. suis* serotype 2 capsular polysaccharide (CPS) monoclonal antibody (mAb) (C) or an anti-tetanus toxoid (TT) mAb (D). Lane 1, 10 µl of SeeBlue Plus 2 Prestained Protein standard (Ladder; Invitrogen); Lane 2, 10 µg of TT purified monomer; Lane 3, 10 µg of the 2:1 mixed conjugate; Lane 4, 10 µg of the 1:1 mixed conjugate; Lane 5, 10 µg of free depolymerized *S. suis* serotype 2 CPS.

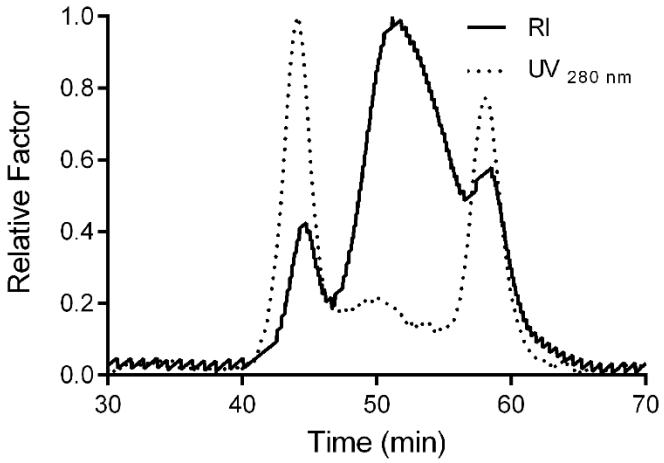


Figure 2. HPLC elution profile of the 2:1 mixed conjugate.

Differential refractometer (RI; full line) and UV detection (UV $_{280\ nm}$; dotted line) profiles are shown. Similar profiles were observed for the 1:1 mixed conjugate.

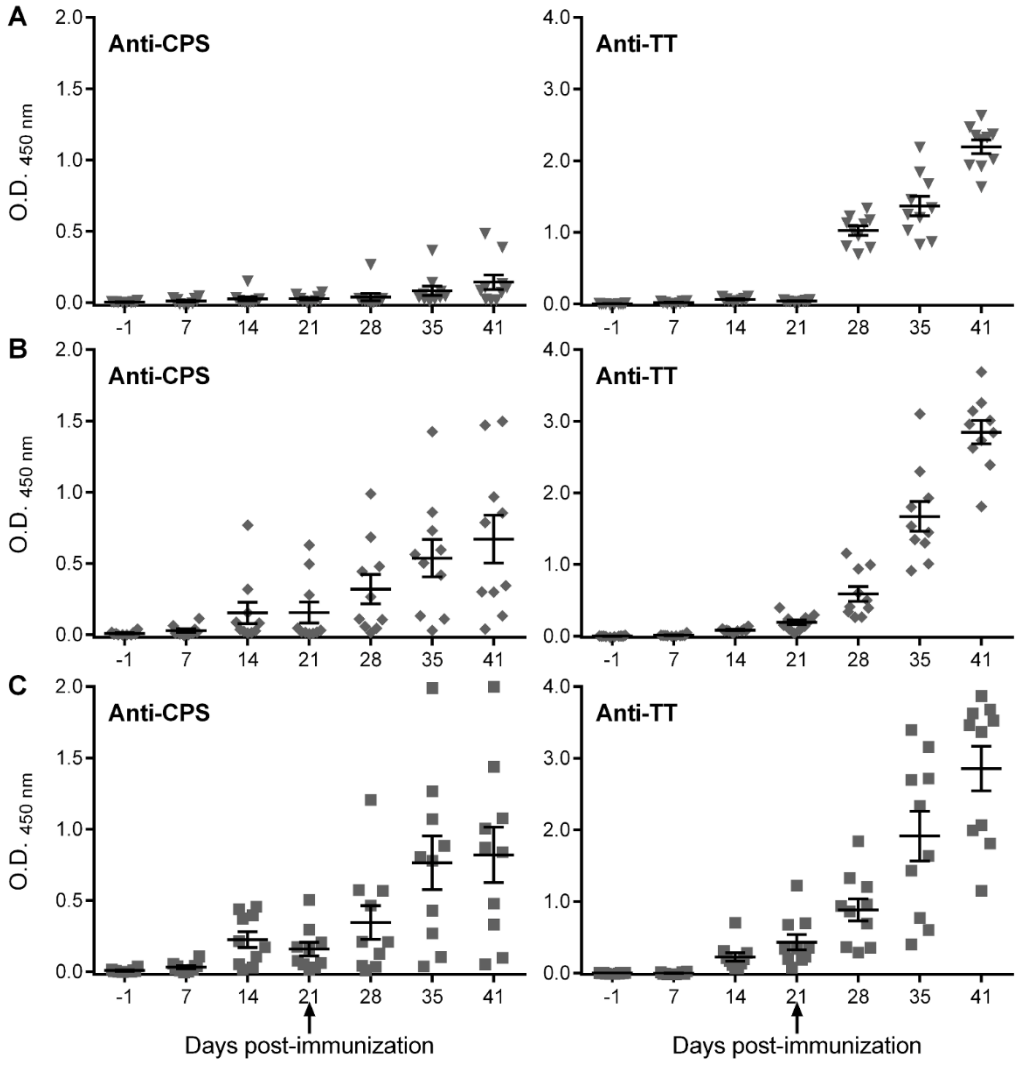


Figure 3. Kinetics of total antibody responses of mice immunized with 25 µg of the 2:1 mixed conjugate adjuvanted with either CpG (A), STIMUNE® (B), or TiterMax Gold® (C).

Mice (n = 10) were immunized on day 0 and boosted on day 21.ELISA plates were coated either with native capsular polysaccharide (CPS) or tetanus toxoid (TT) and incubated with blood samples diluted 1:100 or 1:20,000 to measure anti-CPS and anti-TT antibodies, respectively. Total (IgG+IgM) antibody levels are shown for individual mice, with horizontal bars representing mean ± SEM of O.D. $_{450nm}$ values. Arrow at day 21 indicates boost. To simplify the graph, kinetics for the respective placebo groups (which were all negative) are not shown.

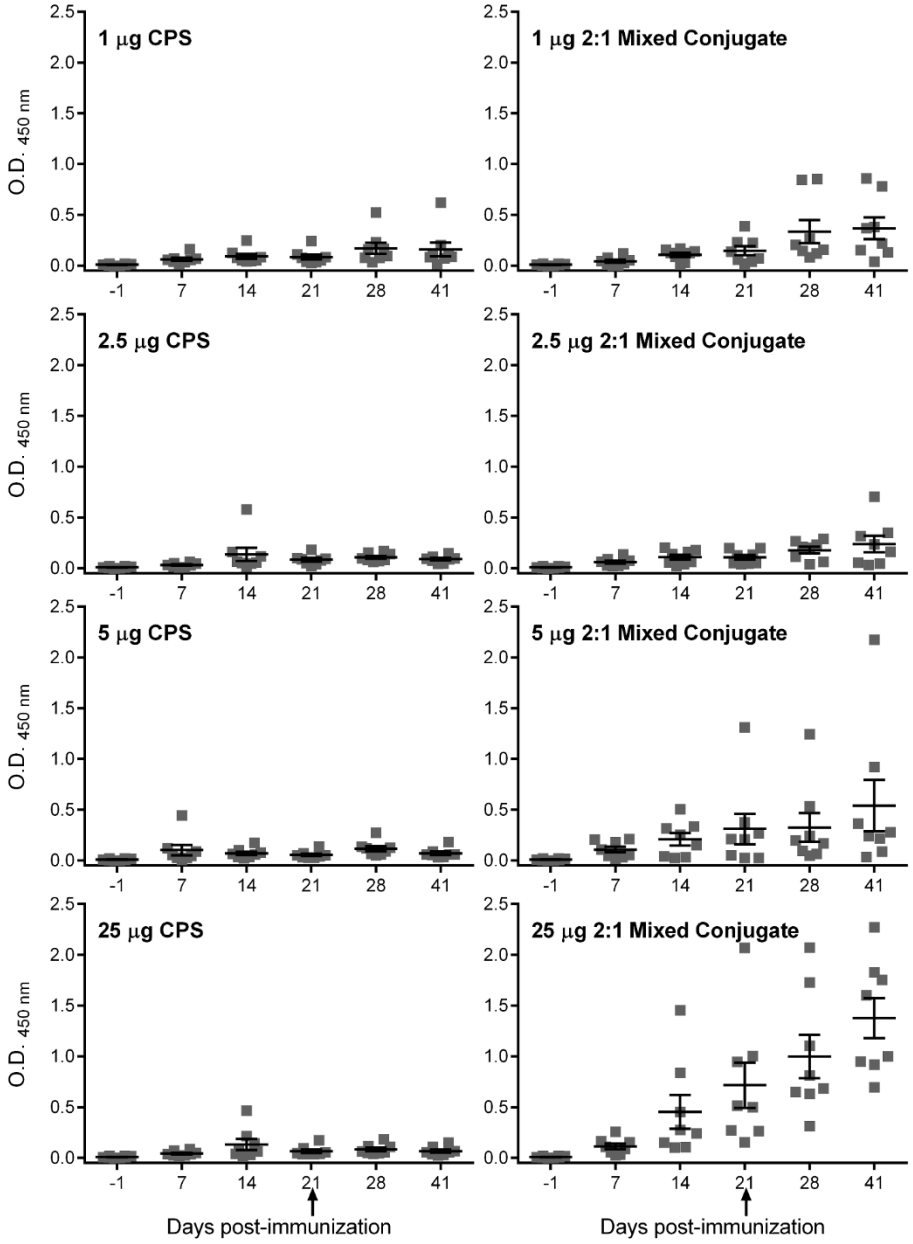


Figure 4. Dose-response effect on total antibody levels of mice immunized with either free depolymerized capsular polysaccharide (CPS) or 2:1 mixed conjugate at 1, 2.5, 5 or 25 μg adjuvanted with TiterMax Gold®.

Mouse groups (n = 8) were injected on day 0 and boosted on day 21. ELISA plates were coated with native CPS and incubated with blood samples diluted 1:100. Total (IgG+IgM) anti-CPS antibody levels are shown for individual mice, with horizontal bars representing mean ± SEM of O.D. $_{450nm}$ values. Arrow at day 21 indicates boost.

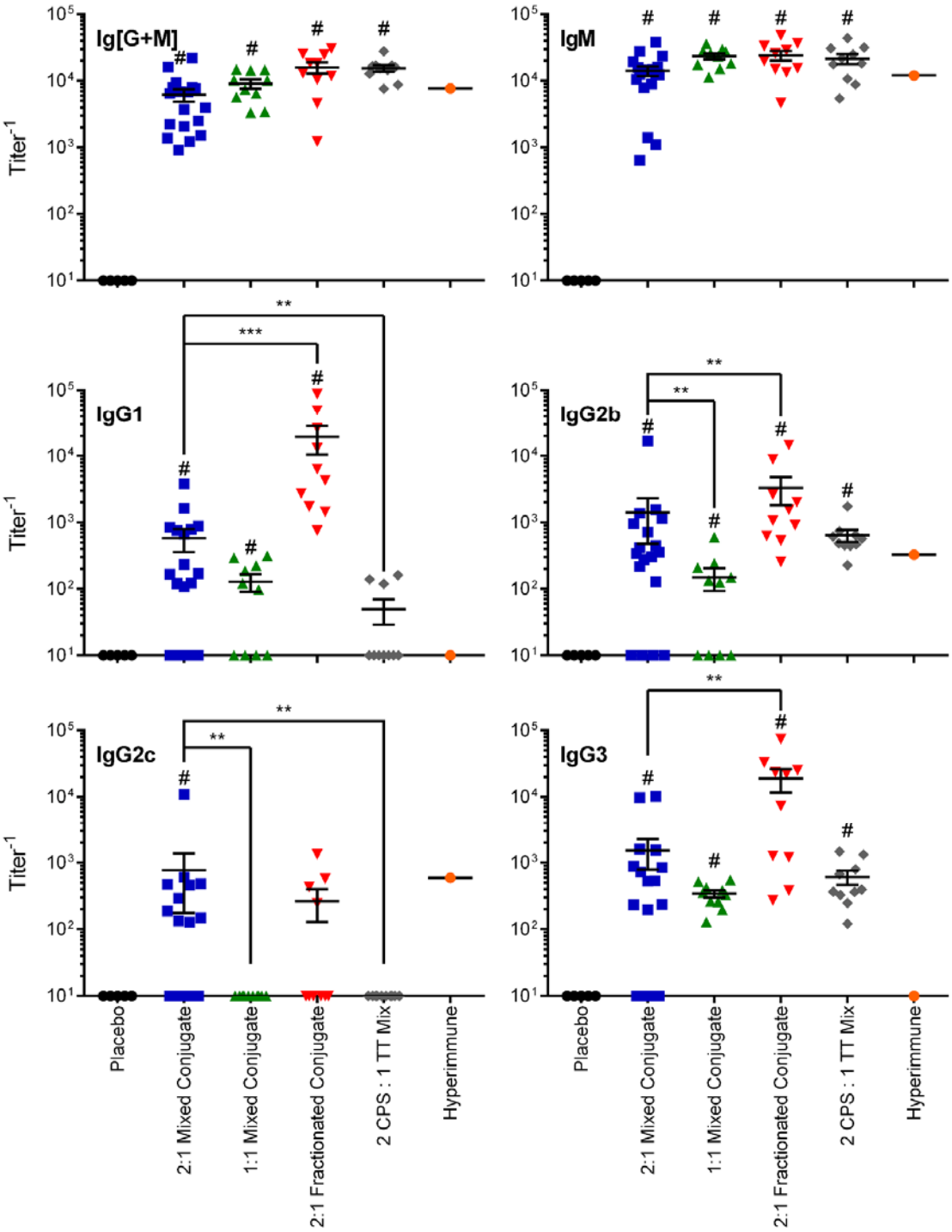


Figure 5. Titers of different anti-CPS antibody isotypes in mice immunized with conjugate vaccines adjuvanted in TiterMax Gold®.

Mouse groups were as follows: placebo (n = 5), 2:1 mixed conjugate (n = 18, animals from the 2 previous immunizations with 25 μg), 1:1 mixed conjugate (n = 10), 2:1 fractionated conjugate (n = 10), 2 CPS: 1 TT unconjugated control mixture (n = 10). All mice were immunized with 25 μg of antigen in TiterMax Gold® on day 0, boosted on day 21 and sera collected at day 42. A pool of hyperimmune mouse sera from 6 mice was also included for comparative purposes. For the titration, ELISA plates were coated with native CPS and incubated with two-fold serial dilutions of sera. Isotypes were detected using specific HRP-conjugated anti-mouse Ig[G+M], IgM, IgG1, IgG2b, IgG2c or IgG3 antibodies. Titers for individual mice are shown, with horizontal bars representing mean ± SEM. # denotes titers significantly different than those of the placebo group ($P < 0.05$), while differences between other groups are denoted as: **, $P < 0.01$ and ***, $P < 0.001$. Abbreviations: CPS, capsular polysaccharide from *S. suis* serotype 2; TT, tetanus toxoid.

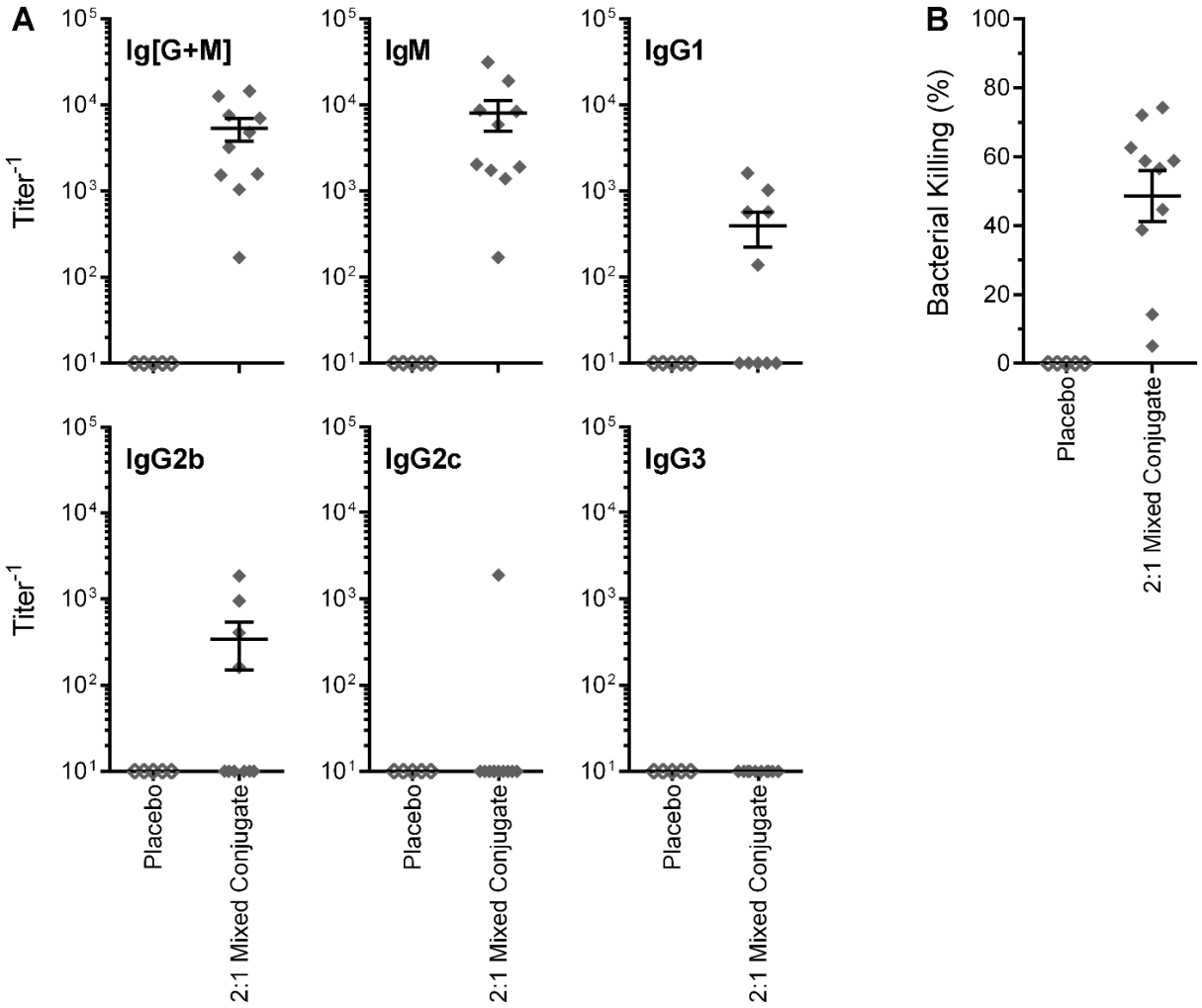


Figure 6. Isotyping and functional studies of antibodies induced in mice immunized with 2:1 mixed conjugate vaccine in STIMUNE®.

Mice (n = 10) were immunized on day 0 and boosted on day 21 with 25 µg of the 2:1 mixed conjugate adjuvanted with STIMUNE®. Placebo mice (n = 5) were similarly injected with PBS adjuvanted with STIMUNE®. Sera were collected on day 42. (A) For titration of anti-CPS antibody isotypes, ELISA plates were coated with native CPS, incubated with two-fold serial dilutions of sera, and isotypes detected using specific HRP-conjugated anti-mouse Ig[G+M], IgM, IgG1, IgG2b, IgG2c or IgG3 antibodies. (B) Opsonophagocytosis killing of *S. suis* type 2 strain S735 by day 42-sera from mice immunized with 25 µg of the 2:1 mixed conjugate adjuvanted with STIMUNE®. A 40 % (v/v) of sample serum and a bacterial MOI of

0.1 were added to fresh whole-blood from naïve mice to perform the assay. Viable bacterial counts were performed after 2 h of incubation. To determine bacterial killing, viable bacterial counts from tubes incubated with sample sera were compared to those incubated with control naive mouse sera. Results are expressed as % of bacterial killing for individual mice, with horizontal bars representing mean ± SEM. Abbreviations: CPS, capsular polysaccharide from *S. suis* serotype 2.

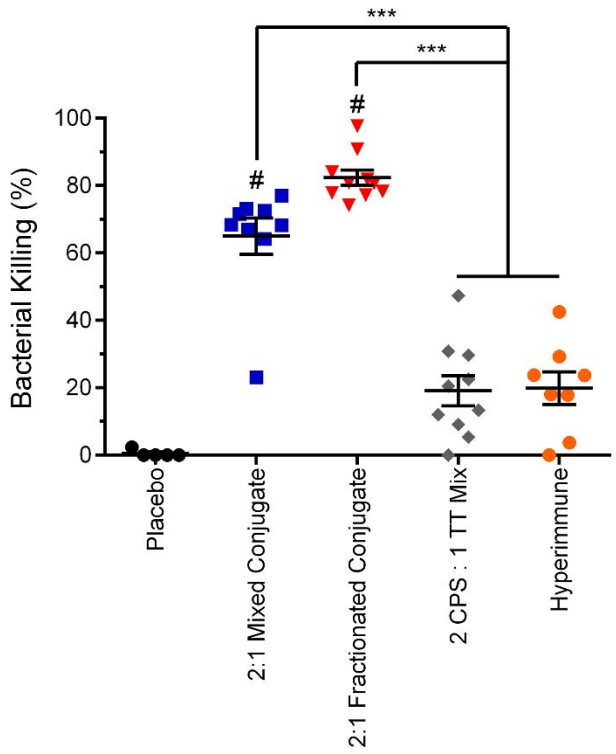


Figure 7. Opsonophagocytosis killing of *S. suis* type 2 strain S735 by day 42-sera from mice immunized with different CPS conjugate vaccines adjuvanted with TiterMax Gold®.

Mouse groups were as follows: placebo (n = 5), 2:1 mixed conjugate (n = 10), 2:1 fractionated conjugate (n = 10), 2 CPS: 1 TT unconjugated control mixture (n = 10). All mice were immunized with 25 µg of antigen in TiterMax Gold® on day 0, boosted on day 21 and sera collected at day 42. A pool of hyperimmune mouse sera from 6 mice was also included for comparative purposes (n = 8 experimental replicates). A 40 % (v/v) of sample serum and a bacterial MOI of 0.1 were added to fresh whole-blood from naïve mice to perform the assay. Viable bacterial counts were performed after 2 h of incubation. To determine bacterial killing, viable bacterial counts from tubes incubated with sample sera were compared to those incubated with control naive mouse sera. Results are expressed as % of bacterial killing for individual mice, with horizontal bars representing mean ± SEM. # denotes values significantly different than those of the placebo group ($P < 0.01$), while differences between other groups are denoted as: ***, $P < 0.001$. Abbreviations: CPS, capsular polysaccharide from *S. suis* serotype 2; TT, tetanus toxoid.

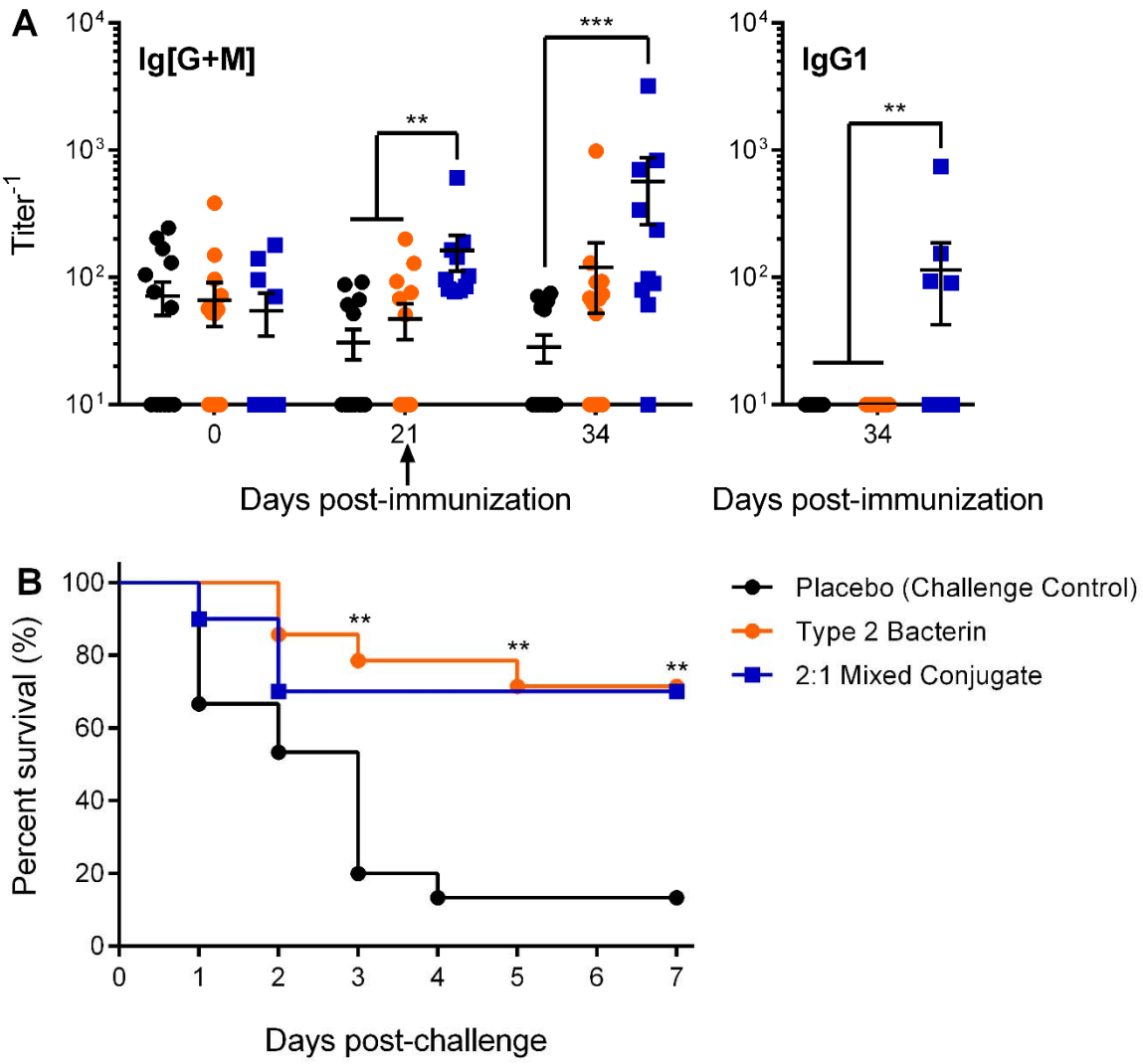


Figure8. Immunogenicity and protection studies in pigs.

Animals were blocked by litter and then randomly assigned to one of four groups: group 1, n = 14; group 2, n = 10, group 3, n = 15; and group 4, n = 5. Groups 1-4 were commingled until group 4 (strict control) was removed at study day 35. Blood samples were collected on study days 0, 21 and 34 for determination of serum antibody levels. The piglets were injected intramuscularly twice at a 3-week interval (study day 0 and 21) with 2 ml of the respective vaccine or placebo adjuvanted with STIMUNE®: group 1 was vaccinated with adjuvanted *S. suis* type 2 bacterin, group 2 was injected with the adjuvanted 2:1 mixed conjugate, group 3 was given 2 ml of adjuvanted PBS. (A) Kinetics of serum antibody response of immunized pigs. ELISA plates were coated with native capsular polysaccharide, incubated for 1 h with two-fold serial dilutions of sera, and isotypes were detected using specific HRP-conjugated

anti-pig Ig[G+M] or IgG1 antibodies. Antibody titers for individual pigs are shown, with horizontal bars representing mean ± SEM. Arrow at day 21 indicates boost. **, $P < 0.01$ and ***, $P < 0.001$ as determined by one-way ANOVA (B) Protection study. On day 36, groups 1-3 were challenged intraperitoneally with 3 x 10^9 CFU/dose of *S. suis* type 2 isolate ATCC 700794. Following challenge, pigs were monitored daily over a period of seven days for the presence of clinical signs. Note: on day 21, one animal from the bacterin group was euthanized due to complications following serum collection, which leaves (n = 14) at day 34 and for the challenge. **, $P < 0.01$ for both bacterin- and 2:1 mixed conjugate-vaccinated groups compared to placebo (challenge control) group.

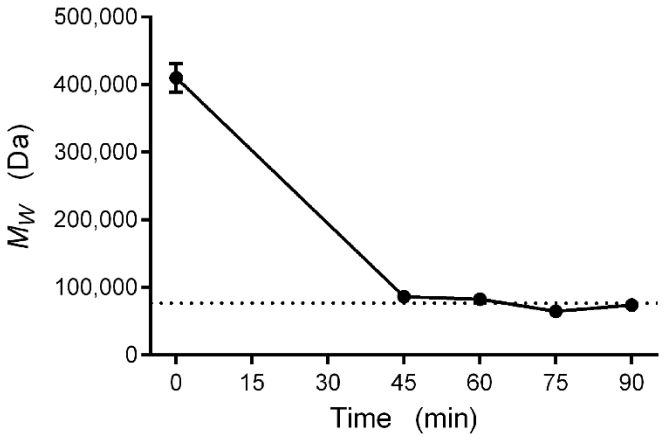


Figure S1. Depolymerization of *S. suis* type 2 capsular polysaccharide (CPS) by ultrasonic irradiation.

Samples of CPS were taken at different time points and were analyzed by size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) in order to determine the weight-average molar mass (M_w). After 60 min, the M_w plateaued, as illustrated by the dotted line.

ARTICLE VII

Characterization and protective activity of monoclonal antibodies directed against *Streptococcus suis* serotype 2 capsular polysaccharide obtained using a glycoconjugate vaccine

---

Guillaume Goyette-Desjardins [1,2,3,4], Sonia Lacouture [1,2], Jean-Philippe Auger [1,2,3], René Roy [2,3,4], Marcelo Gottschalk [1,2,3,4], and Mariela Segura [1,2,3,4]

[1] Research Group on Infectious Diseases in Production Animals, Faculty of Veterinary Medicine, University of Montreal, St-Hyacinthe, Quebec, Canada

[2] Swine and Poultry Infectious Diseases Research Centre, Quebec, Canada

[3] Pharmaqam, Université du Québec à Montréal, Montreal, Quebec, Canada

[4] Canadian Glycomics Network (GlycoNet), University of Alberta, Edmonton, Alberta, Canada

Sous révision dans *Glycobiology*. **Avril 2019**. (#GLYCO-2018-00147)

Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai participé activement à la conception de l'étude, à la réalisation des expériences (75%), à l'analyse des résultats et à l'écriture de l'article.

Abstract

Streptococcus suis serotype 2 is an encapsulated bacterium and an important swine pathogen causing septicemia and meningitis. Opsonizing antibody responses targeting capsular polysaccharides (CPSs) are protective against extracellular pathogens. To gain new insights into the protective activity of monoclonal antibodies (mAbs) directed against *S. suis* serotype 2 CPS, mAbs were obtained by immunizing BALB/c mice with a glycoconjugate vaccine made from *S. suis* serotype 2 CPS. Three new hybridomas were isolated; of which, two were murine IgMs and the other a murine IgG1. Whereas the IgMs (mAbs 9E7 and 13C8) showed different reactivity levels with *S. suis* serotypes 1, 1/2, 2 and 14, the IgG1 (mAb 16H11) was shown to be specific for serotype 2 CPS only. Nevertheless, all mAbs targeted the sialylated chain of the CPSs. Using an opsonophagocytosis assay, the IgMs were found to be opsonizing towards the *S. suis* serotypes to which they cross-react, while the IgG1 failed to induce bacterial elimination. In a model of passive immunization of CD-1 mice followed by a lethal challenge with *S. suis* serotype 2, the IgG1 (mAb 16H11) and IgM cross-reacting only with serotype 14 (mAb 13C8) failed to protect, while the IgM cross-reacting with serotypes 1, 1/2, and 14 (mAb 9E7) was shown to be protective. Protection during the lethal challenge was associated with a reduction in bacteremia levels at 24 and 48 h post-infection. These new mAbs show promises as new diagnostic tools for *S. suis* identification, as well as potentials for therapeutic applications.

Introduction

Streptococcus suis is an encapsulated Gram-positive bacterium and one of the most important bacterial pathogen in the porcine industry, resulting in important economic losses (1). To date, 35 *S. suis* serotypes have been described based on the capsular polysaccharide (CPS) antigenic diversity. *S. suis* serotype 2 is considered the most virulent, being the serotype most frequently isolated from clinical samples and associated with disease in pigs in most countries (2). *S. suis*, mainly serotype 2, is also an important emerging zoonotic agent for people in close contact with pigs or pig-derived products (2). Of the various manifestations of the disease, septicemia and meningitis are by far the most striking, but other clinical outcomes can also be observed (1). Research has been ongoing for decades in the hope of developing an efficient commercial vaccine to protect post-weaning pigs against *S. suis* disease. Yet, to our knowledge, no such vaccine with proven efficacy is available (3).

It is well known that the thick-surface associated CPS confers protection to *S. suis* against the immune system, notably by resisting phagocytosis (4, 5). Thus, as with other encapsulated pathogens such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae*, and Group B *Streptococcus*, antibodies directed against the CPS are highly opsonizing and protective (6, 7). Paradoxically, due to their carbohydrate nature, CPSs are generally considered poorly immunogenic since they are unable to recruit T cell help for B cell functions (8). A well known strategy to overcome the T-independent (TI) nature of CPSs is to conjugate the polysaccharide onto a carrier protein that will provide T-dependent (TD) epitopes to allow a potent vaccinal response against the polysaccharide (9, 10). Thus, we recently created a first glycoconjugate vaccine, made from *S. suis* serotype 2 CPS coupled to tetanus toxoid (TT) by reductive amination, and found it to induce opsonizing anti-CPS antibodies in mice and to be protective in pigs against a challenge carried out with this serotype (11).

Currently, exact structures for the repeating units (RUs) of the CPS of seven different serotypes have been reported, including those for serotypes 2, 14, 1, 1/2, and 9 of *S. suis* (12-16). Serotypes 2, 14, 1, and 1/2 RUs are formed of acidic branched hexa- or heptasaccharides

with α2,6-linked sialic acid (Neu5Ac) at their non-reducing ends as a common feature (Fig. 1). Serotype 9 RU is formed of an acidic branched tetrasaccharide and non-sialylated (Fig. 1). Serotypes 2 and 1/2 and serotypes 1 and 14 share common epitopes and present cross-reactions by co-agglutination (2). These cross-reactions cannot be resolved by PCR since, as expected, these serotypes do not possess unique *cps* genes (2, 17). Indeed, serotypes 2 and 14 both possess a β-galactose (Gal) in their side chain that is found *N*-acetylated (GalNAc) in serotypes 1 and 1/2 (14). More recently, it has been shown that a single amino acid polymorphism in the glycosyltransferase CpsK defines the enzyme substrate predilection for Gal or GalNAc and therefore determines CPS composition, structure and strain serotype (18, 19). Still, very little is known about *S. suis* type 2 CPS protective epitopes. A previous study aimed at explaining the serological characteristics of *S. suis* serotypes 2, 1, 1/2, and 14 using purified CPSs and rabbit type-specific sera showed that the sialic acid-bearing side chain and, most importantly, the terminal sialic acid, constitutes an important immunogenic structure for serotype 2 CPS (14).

Monoclonal antibodies (mAbs) are nowadays common useful tools employed in many settings, ranging from the study of bacterial virulence factors to therapeutic applications. Previously, Charland *et al.* reported the characterization and protective activity of mAb Z3 which was found to be directed to the CPS of *S. suis* serotype 2; interestingly, it also reacted with the CPS of serotypes 1 and 1/2 (20). In that study, although more than 3000 clones were tested following hyperimmunization of mice with formaldehyde-inactivated bacteria, only the mAb Z3 was found to react with the CPS, which suggests a very low frequency of CPS-specific clones. The mAb Z3 was also shown to present a specificity for the terminal sialic acid (20). It has also been well demonstrated that *S. suis* serotype 2 CPS is non-immunogenic, even when expressed at the bacterial surface during an infection or in the presence of strong adjuvants such as water-in-oil emulsions like TiterMax Gold® and STIMUNE® (11, 21-23).

Our hypothesis was that a glycoconjugate (made from *S. suis* serotype 2 CPS coupled to TT) improves obtention and diversity of serotype 2 CPS-specific B cell clones and thus hybridomas after fusion with a myeloma cell line. Therefore, the aim of this study was to obtain, characterize and study the protective activity of additional murine mAbs targeting *S.*

suis serotype 2 CPS. In turn, these new mAbs were also used to help define the protective epitopes of *S. suis* serotype 2 CPS.

Results

Characterization of mAbs

The screening steps against *S. suis* type 2 CPS of mAb production by ELISA led to the selection of 3 positive clones: 9E7 (IgM), 13C8 (IgM), and 16H11 (IgG1) out of 700 clones tested. In addition, previously described mAb Z3 was included in this study as a protective positive control and for dot-blot experiments regarding CPS specificity (20). Although it had originally been reported to be an IgG2b, mAb Z3 was found later to be of the IgM isotype (M. Gottschalk, unpublished data); this discrepancy could be explained by the low specificity of the commercial reagents that were available at the time of the study by Charland *et al.* (20).

In order to evaluate the avidity of our mAbs toward type 2 CPS, a thiocyanate elution assay was performed using an anti-CPS indirect ELISA. As is shown in Fig. 2, the mAb with the strongest antibody avidity index (AI) was found to be the IgG1 16H11, followed closely by the IgM 9E7, while IgMs 13C8 and Z3 showed intermediate and low avidities, respectively.

Characterization of the S. suis capsular epitopes recognized by the mAbs

Since mAb Z3 was originally reported to recognize the sialylated CPSs of *S. suis* serotypes 1, 1/2 and 2 (20), we decided to conduct dot-blot analyses using both heat-killed bacteria and purified CPSs of those serotypes but also serotype 14 of *S. suis*, since their CPS RU structures are highly similar (Fig. 1). As aforementioned, they all possess sialic acid and are known to present cross-reactions when serotyping is performed with rabbit antisera (14). Serotype 9 was included as a negative control since its CPS RU structure is unrelated to those of the other studied serotypes (Fig. 1). Results of the dot-blots analyses are shown in Fig. 3. Additionally, densitometric analysis of the dot-blot results was performed to distinguish between strong and weak reactions of a single mAb towards the different serotypes (Fig. 4).

As reported (20), IgM mAb Z3 strongly recognized the CPSs of serotypes 1, 1/2, and 2, but not that of serotype 14 (Fig. 3A and 4A). In contrast, IgM mAb 13C8 gave strong reactions with serotypes 2 and 14, but did not recognize the CPSs of serotypes 1 and 1/2 (Fig. 3B and 4B). The IgM mAb 9E7 recognized all four CPSs (1, 1/2, 2, and 14) (Fig. 3C and 4C). Interestingly, IgG1 mAb 16H11 reacts solely with serotype 2 (Fig. 3D and 4D).

The cross-reactions between *S. suis* serotypes 2 and 14 (IgM mAbs 13C8 and 9E7) were unexpected based on published serological tests (21, 24), especially in the absence of reactions with serotypes 1 and 1/2 as is the case for mAb 13C8. Western blots using whole bacterial cell extracts showed that the serotype 14 reaction of mAbs 13C8 and 9E7 was specific toward the CPS, thus ruling out potential protein contamination of the CPS preparation (data not shown). ELISA screening and dot-blot analyses showed for all mAbs a strong specificity toward sialylated CPSs but not against their desialylated counterparts (data not shown), as previously reported for IgM mAb Z3 (20). Finally, none of the mAbs recognized neither the heat-killed bacteria nor the CPS of serotype 9, used as control (Fig. 3 and 4).

Functional activity of mAbs

Opsonophagocytosis is thought to be the main mechanism responsible for clearing encapsulated Gram-positive bacteria via both Fc-receptor-mediated and/or C1q- and/or C3b-mediated (complement-dependent) phagocytosis (25-28). Therefore, the functional activity of the mAbs against *S. suis* serotype 2 was investigated by performing a standardized opsonophagocytosis assay (OPA) test using whole blood from mice as effector cells (11, 29). Firstly, a dose-response experiment using purified mAbs Z3, 13C8, 9E7 and 16H11 was performed (Fig. 5). All IgMs (namely mAbs Z3, 13C8 and 9E7) were found to be opsonizing, with a maximum killing value of approximately 50-60% at the highest concentration used. These data indicate that these purified mAbs are functional and able to mediate the clearance of *S. suis* serotype 2 by opsonophagocytosis *in vitro*. Since the opsonizing capacity of IgM mAb 13C8 dropped faster than that of IgM mAbs Z3 and 9E7, opsonic indexes (OIs), corresponding to the dose of mAb (in µg/ml) yielding 50% of maximal killing value, were calculated. The mAb 13C8 has an OI of 27.8 µg/ml, an opsonizing capacity approximately 6

to 12 times lower than that of mAb 9E7 (OI = 4.35 µg/ml) or mAb Z3 (OI = 2.35 µg/ml), respectively. In contrast to IgMs, murine IgG1 mAb 16H11 was found to be not opsonizing, with killing values below 5 %, even with a higher dose of 500 µg/ml (Fig. 5 and data not shown).

Since the three IgMs were opsonizing with *S. suis* serotype 2, we wanted to validate whether the cross-reactions observed by dot-blot with the CPSs of other serotypes (Fig. 3 and 4) were specific and of clinical importance. To this end, OPAs were repeated using a single dose of 125 µg/ml of mAb against strains of serotypes 1, 1/2 and 14. A serotype 9 strain and an IgM isotype control were included as negative controls. As shown in Fig. 6, OPA results correlated with cross-reactions observed in dot-blot analyses. The IgM mAb Z3 induced opsonophagocytosis of *S. suis* types 1, 1/2 and 2 but not of type 14. The IgM mAb 13C8 opsonized types 2 and 14 only; while IgM mAb 9E7 opsonized all 4 serotypes. The absence of significant killing ($P > 0.05$) by either the isotype control (Fig. 5 and 6) or by mAb-opsonization of *S. suis* serotype 9 (Fig. 6E) demonstrated the specificity of these mAbs toward the sialylated CPSs of *S. suis* serotypes 2, 1, 1/2, and/or 14.

Additionally, the ability of these mAbs to induce bacterial agglutination was also investigated, since this confounding mechanism could also lead to a positive result in the OPA assay (Fig. 7). The agglutination assay was performed in the same fashion as the OPA assay, minus the whole blood (source of phagocytic cells). Both highly opsonic IgM mAbs Z3 and 9E7 produced a significant reduction in the viable counts of the *S. suis* serotype 2 strain ($P < 0.001$), which suggests bacterial agglutination. To the contrary, no reduction in viable counts ($P > 0.05$) was observed with the opsonic IgM mAb 13C8 nor with the nonopsonic IgG1 mAb 16H11, indicating that these two mAbs do not agglutinate *S. suis* serotype 2.

Using J774 murine macrophages and pre-opsonized *S. suis* serotype 2, immunofluorescence studies by confocal microscopy were performed to investigate whether or not the opsonic IgM mAbs can induce immunoclearance of *S. suis* through opsonophagocytosis (Fig. 8). Bacteria were pre-opsonized in the presence of 125 µg/ml of purified mAb and 40 % (v/v) fresh serum from naïve mice as a source of complement. When

S. suis serotype 2 was pre-opsonized by an IgM isotype control, no bacteria could be found inside macrophages (Fig. 8), illustrating well how resistant to phagocytosis encapsulated *S. suis* is (4, 5). When IgM mAbs Z3 and 9E7 were employed to pre-opsonize *S. suis* serotype 2, large bacterial clumps could be found within phagolysosomal (LAMP-1^+) vacuoles (Fig. 8), which was also the case with serotype-specific rabbit antiserum used as positive control. When IgM mAb 13C8 was used for pre-opsonisation, individual bacteria and chains can be observed inside the phagolysosomal vacuoles of macrophages (Fig. 8). Overall, these results indicate that our IgM mAbs do induce opsonophagocytosis *in vitro*, since both bacterial coating and bacterial agglutination by IgM can trigger capture by phagocytes (25, 30).

Protective potential of mAbs

Since the IgM mAbs 9E7 and 13C8 were able to mediate the clearance of *S. suis* serotype 2 by opsonophagocytosis *in vitro*, their protective capacity *in vivo* was evaluated by passive immunization. The IgM mAb Z3 has previously been shown to be protective when used for pre-opsonizing bacteria prior to intraperitoneal infection in mice (20). In this study, to better evaluate the *in vivo* activity of the IgM mAbs, we developed a passive immunization assay in CD-1 mice using intraperitoneal administration of the antibody 1 h prior to a lethal challenge with a LD90 dose of 1 x 10^8 CFU of virulent *S. suis* serotype 2 strain P1/7 intraperitoneally (Fig. 9). In a first pre-trial protection assay, a dose of 20 µg was chosen based on other reports investigating mAbs against *S. pneumoniae* CPSs (31-33). Two days post-infection (p.i.), 9 out of 10 mice in the negative control group presented serious clinical signs of *S. suis* disease and had to be euthanized (Fig. 9A). All mice injected with the polyclonal rabbit serum (used as positive control) survived and did not show any clinical signs. However, no significant protection ($P > 0.05$) was observed in mice passively immunized with either IgM mAbs 9E7, 13C8 or Z3 (the latter included as control) when administrated at a dose of 20 µg (Fig. 9A).

Therefore, we decided to increase the mAb dose to ensure that a failure in protection was not due to a low antibody concentration. In the second protection assay, mice received a dose of 200 µg of IgM mAb. Seven days p.i., all mice from the positive control (rabbit antisera) survived, whereas the IgM isotype control group presented 90 % of mortality (Fig.

9B). Mice injected with IgM mAbs Z3 or 9E7 showed 90 % and 100 % of protection 7 days p.i., respectively. On the other hand, mice treated with IgM mAb 13C8 showed 40 % of protection which was not significantly different than the isotype control ($P = 0.23$). Indeed, at 7 days p.i., protection conferred by IgM mAbs Z3 and 9E7 was found to be significantly higher ($P < 0.05$) than that of IgM mAb 13C8. In agreement with protection levels, at 24 h p.i. mice in the isotype control group showed strong levels of bacteremia that were also sustained 48 h p.i. (Fig. 10). Mice treated with the rabbit antiserum were able to clear the bacteria from circulation as soon as 24 h p.i. (Fig. 10). At 24 h p.i., mice treated with IgM mAbs Z3 or 9E7 showed significantly lower bacteremia than the isotype control group ($P \leq 0.005$) (Fig. 10A). At 48 h p.i., mice treated with IgM mAbs Z3 or 9E7 also presented a considerable reduction in bacteremia compared to the negative control group, although the difference was significant only for IgM mAb 9E7 ($P = 0.027$) (Fig. 10B). In contrast to these two IgMs, bacteremia levels in mice treated with IgM mAb 13C8 were comparable to those of the control group at both time points (Fig. 10).

Discussion

By employing a glycoconjugate made from *S. suis* serotype 2 CPS covalently linked to TT (11), we describe for the first time a murine IgG1 mAb directed to the CPS of *S. suis* serotype 2. Interestingly, this mAb was solely specific for serotype 2 CPS. In addition, this strategy also allowed the obtention of two additional new murine IgMs, each recognizing distinct epitopes of the *S. suis* serotype 2 CPS and cross-reactive epitopes of serotypes 1, 1/2 and/or 14. All reactions were found to be specific towards the sialic acid and/or sialic acid-bearing side chain. Finally, while the IgG1 (mAb 16H11) was unable to promote bacterial immunoclearance *in vitro* or *in vivo* (data not shown), the IgMs were opsonizing and/or agglutinating *in vitro* and/or protective *in vivo*, yet at different degrees (Table I). While it was previously found that a glycoconjugate vaccine made from *S. suis* serotype 2 CPS induces opsonizing anti-CPS antibodies in mice and protected pigs against this serotype (11), in this study it was demonstrated by employing mAbs that the *in vivo* protection is indeed due to the opsonizing capacities of those anti-CPS antibodies. In addition, IgM mAbs that were agglutinating (Z3 and 9E7) were positively associated with protection *in vivo*, as agglutination

contributes greatly to complement activation (see below). Yet, mAb 13C8, which was non-agglutinating and not significantly protective, still was able to trigger phagocytosis of *S. suis* serotype 2 by macrophages and to trigger its killing in our OPA assay. We have thus been able to show that IgM mAbs are opsonizing and lead to bacterial clearance through opsonophagocytosis. In turn, this reflects the great potential of polysaccharides as vaccine antigens in preventing infections by extracellular bacteria.

It is now well known that *S. suis* serotype 2 CPS is particularly nonimmunogenic, even when associated at the bacterial surface in the course of a primary or booster experimental infection (11, 21-23, 34, 35). This low immunogenicity results in low IgM titers and undetectable IgG titers. While one likely explanation is that CPSs are considered as TI antigens, purified CPSs from other streptococci are known to induce not only IgM but also IgG protective antibody responses (23, 36, 37). The low immunogenicity of *S. suis* serotype 2 CPS cannot be explained by the presence of sialic acid, known to possess immunomodulatory properties (23). Nonetheless, as with other encapsulated bacteria, conjugation of CPS to a carrier protein produces a stronger antibody response with TD features such as antibody class switching, affinity/specificity maturation, and immunological memory (8, 9). Here, the use of a glycoconjugate yielded two IgMs and one IgG against *S. suis* serotype 2 CPS, which is an improvement compared to the previous report by Charland *et al.* where whole bacteria was used as immunogen and only one IgM was obtained (20).

The obtained IgG mAb belongs to the IgG1 subclass, known to be privileged during a Th2 immune response. This isotype switch could be favored by the immunization strategy, the mouse genetic background, the immunological properties of the TT protein itself and/or the adjuvant used. For instance, TiterMax Gold® is a squalene-based adjuvant which has been reported to induce either mixed Th1/Th2 or Th2-polarized humoral responses in mice (11, 38, 39). The protein TT has been reported to induce a cytokine profile compatible with a type 2 response (40). Other immunization strategies, such as employing either a different mouse strain, adjuvant or carrier protein for the glycoconjugate, could be used to obtain other IgG subclasses. As TD antibody responses are known to involve processes such as affinity maturation, this could explain why the IgG1-mAb 16H11 possesses such unique specificity to

only *S. suis* serotype 2 CPS and a high avidity. Indeed, murine IgG1 has been previously reported to possess the highest mean antigen-binding affinity of all subclasses (41, 42). Nevertheless, due to its IgG1 nature, this mAb was unable to induce *S. suis* elimination by opsonophagocytosis *in vitro* and thus protection *in vivo* (data not shown). A study using mouse mAbs proposed that the type 1 subclasses (IgG3 >> IgG2b ≥ IgG2a) are superior in both opsonophagocytosis activity and complement activation than the type 2 (IgG1) subclass (41, 43). Nevertheless, some mouse IgG1 mAbs against bacterial CPSs have been reported to be opsonic and/or protective against *S. pneumoniae* and Group B *Streptococcus* (33, 44). How these mouse IgG1 can mediate protection is still the subject of ongoing research, and some new mechanisms are emerging, such as immunomodulatory functions (45) and bacterial gene expression alterations (46).

On the other hand, the two newly reported IgMs (mAbs 9E7 and 13C8) were shown to be opsonizing *in vitro*. However, in our passive immunization model, mAb 9E7 protects against challenge with a lethal dose of *S. suis* serotype 2, while mAb 13C8 confers no significant protection. This difference is also reflected in their respective OIs, but, surprisingly, does not correlate with their difference in avidity. Indeed, mAbs Z3 (low avidity) and 9E7 (strong avidity) are both highly opsonizing and protective while mAb 13C8 (intermediate avidity) is weakly opsonizing and non-protective in the conditions used. In the literature, correlation of protection with antibody avidity was reported for IgG, not for IgM (32, 47-49). A more likely explanation regarding the difference in behavior between mAbs 9E7/Z3 and mAb 13C8 may be their differing epitope preferences: the 'poly-reactivity' of mAbs 9E7/Z3 might explain their ability to agglutinate *S. suis* serotype 2. As a matter of fact, agglutination by antibodies (notably IgM, but also IgG and IgA) greatly facilitates the removal of foreign pathogens and helps prevent the establishment of colonization and/or infection (30, 50). A study demonstrated how *S. pneumoniae* minimizes chain length to evade complement activation and gain advantage in a model of systemic infection, and how this effect can be overcome by antibody agglutination (51). Other studies of pneumococcal agglutination using an IgM mAb have also suggested that complement is involved in bacterial immunoclearance by phagocytes and required for protection during *in vivo* challenges (31, 52, 53). This bacterial clearance is indeed dependent of complement receptor 3 (CR3) as well as C3, while being

independent of the Fcα/μ receptor (54). Thus, it becomes clear that IgM-coated bacteria and agglutinated bacteria (with either IgM, IgG or IgA) can trigger complement-dependent phagocytosis.

In the case of *S. suis*, while the precise epitopes to which the mAbs bind remain unknown, the differences in their functional characteristics *in vitro* and *in vivo* and their binding specifies to the different *S. suis* serotypes provide evidence that only certain CPS determinants might elicit protective antibodies; a crucial question to be answered in vaccine development. Since all mAbs were specific toward the sialylated CPSs, these findings demonstrate a dominance of the sialic acid-bearing side chain for the antibody response. This is in agreement with previous studies reporting that desialylated type 2 CPS is only weakly recognized by reference rabbit antiserum and that described serological cross-reactions between other sialylated serotypes (1, 1/2 and 14) would be mainly related by structural/conformational control by sialic acid (14, 55). The difference in specificity between mAbs 9E7 and 13C8 could possibly be an example of the influence played by the substitution of the β-Gal by a β-GalNAc in serotypes 1 and 1/2; whether this effect reflects the recognition of a structural or conformational epitope remains to be determined. Further studies will be required to allow exact determination of the epitopes recognized by these mAbs, and of the importance of each constituent of *S. suis* type 2 CPS. Nevertheless, our data indicates that antibodies targeting the sialic acid-bearing side chain ‘‘epitope’’ are protective against *S. suis* serotype 2.

In conclusion, using mice immunized with a glycoconjugate vaccine made from *S. suis* serotype 2 CPS, three new B cell hybridomas were isolated: of these mAbs, two are IgMs and one is an IgG1 with different specificities against *S. suis* sialylated CPSs. Also, it was demonstrated that a glycoconjugate vaccine can induce protective antibodies against *S. suis* serotype 2 CPS that mediate *in vivo* bacterial killing by opsonophagocytosis. Thus, due to their described properties, these mAbs show potential for diagnostic and/or therapeutic purposes and provide fundamental knowledge of the CPS immunogenic determinants to further advance the development of optimized glycoconjugate vaccine against *S. suis*. By extension, the results presented herein provide new insights into the recognition of carbohydrate antigens by specific

antibodies and how these properties can translate into protective immunity against encapsulated pathogens.

Material and Methods

Bacterial strains and culture conditions

S. suis strains that were used in this study are as follows: serotype 2 strain S735; serotype 1 strain 1178027; serotype 1/2 strain 2651; serotype 14 strain DAN13730; and serotype 9 strain 1273590. All these strains were previously used for CPS structure determination (12-15). They were included here as the target strains for *in vitro* opsonophagocytosis assays (OPA), agglutination test, to prepare the heat-killed bacteria used in the dot-blot analyses and to purify the CPSs. *S. suis* serotype 2 strain P1/7 was used for the protection assay. Isolated colonies on sheep blood agar plates were inoculated in 5 ml of Todd-Hewitt broth (THB) (Becton Dickinson, Franklin Lakes, NJ) and incubated for 8 h in a water bath at 37°C with agitation at 120 rpm. Working cultures were prepared by transferring 10 µl of 8 h-cultures diluted 1:1,000 with phosphate-buffered saline (PBS, pH 7.3) into 30 ml of THB, which was incubated for 16 h. Overnight cultures were washed twice with PBS and then resuspended in 30 ml of PBS (heat-killed bacteria and opsonophagocytosis assay) or THB (mouse protection assay). Bacterial solutions were appropriately diluted, and plated on Todd-Hewitt broth agar (THA, Becton Dickinson) to accurately determine bacterial concentrations using an Autoplate 4000 Spiral Plater (Spiral Biotech, Norwood, MA). Heat-killed bacteria cultures were obtained as previously described (56). Briefly, overnight cultures were washed twice with PBS and then resuspended in 30 ml of PBS. A sample was taken to perform bacterial counts on THA. Bacteria were immediately killed by incubating at 60°C for 45 min and then were cooled on ice. Bacterial killing was confirmed by absence of growth on blood agar for 48 h.

Capsular polysaccharide purification and mild acid hydrolysis

CPSs from *S. suis* serotypes 1, 1/2, 2, 14, and 9 were purified as previously described (12-15). Except for serotype 9, desialylated CPSs were prepared by mild acid hydrolysis, and desialylation was confirmed by nuclear magnetic resonance, as previously described (12-14).

Monoclonal antibody production

All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals by the Animal Welfare Committee of the University of Montreal. BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were immunized subcutaneously at day 0 with 25 µg of the CPS-TT glycoconjugate emulsified 1:1 (vol/vol) with TiterMax Gold®, as described (11). Booster injections of 5 µg of the glycoconjugate emulsified with TiterMax Gold® were given on days 21 and 35. Three days after the last booster immunization, mice were serologically tested with the indirect ELISA and the spleen of the mouse showing the highest reaction (Total IgG plus IgM anti-CPS titer of 4,021) was recovered and fused with myeloma cell line SP2/0 using 50% (w/v) PEG 1500 (Sigma-Aldrich, Oakville, ON, Canada) as a fusogen (57). Hybridoma supernatants were screened for the presence of anti-serotype 2 CPS antibodies using an indirect ELISA (see below). Positive hybridomas were cloned and then used to produce mAbs-containing supernatant. The immunoglobulin class of the mAbs in the culture supernatant was determined by anti-CPS ELISA titration using specific HRP-conjugates (see below).

Monoclonal antibody purification

Mouse IgM mAbs (Z3, 9E7, and 13C8) were purified by gel filtration chromatography using a XK16-100 column packed with Superdex 200 PG (GE Healthcare Life Sciences, Uppsala, Sweden), as described by Hale (58). Firstly, culture supernatant containing mouse IgM was concentrated by 3 cycles of precipitation with ammonium sulfate, stirred and centrifuged. The precipitate was resuspended in PBS, injected unto the gel filtration column and eluted with 0.02 M $NaHPO_4$ (pH 7.2), 0.15 M NaCl. The IgM mAbs (approx. 950 kDa) eluted in the fractions corresponding to the void volume.

Mouse IgG1 mAb (16H11) was purified by affinity chromatography using Protein A Sepharose CL-4B resin (GE Healthcare Life Sciences). The column was equilibrated in binding buffer (0.5 M glycine/NaOH [pH 9.0], 3 M NaCl). Culture supernatant containing mouse IgG1 was mixed 1:1 with binding buffer, then applied to the column. Since protein A has a weak interaction with mouse IgG1, elution was carried in two steps. For the first step,

elution was performed using 0.1 M sodium citrate (pH 5.5), where mouse IgG1 eluted, followed by a second step with 0.2 M glycine/HCl (pH 2.5) to remove all bound material. All eluted fractions were neutralized using 1 M Tris/HCl (pH 9.0).

Purified fractions containing anti-CPS mAbs (as identified by the indirect ELISA) were buffer-exchanged with PBS and concentrated using Amicon Ultra centrifugal filters (30K; EMD Millipore, Billerica, MA, USA). Finally, purified mouse immunoglobulins were quantified by direct ELISA (see below).

Direct ELISA for mouse immunoglobulin (Ig) quantification

To determine the amount of mouse Ig in either culture supernatants or purified fractions, samples were serially diluted with PBS and added to wells of an ELISA plate (Nunc-Immuno Polysorp; Canadawide Scientific, Toronto, ON, Canada). Standard curves ranging from 0 – 50 ng/ml were prepared in the same manner using either IgM or IgG1 isotype controls (Southern Biotech, Birmingham, AL). After overnight coating at 4°C, plates were washed with PBS containing 0.05% (vol/vol) Tween 20 (PBST) and blocked with PBS containing 1% (wt/vol) bovine serum albumin (BSA) (HyClone Laboratories, Logan, UT) for 1 h. After washing, the plates were incubated for 1 h at room temperature with an HRP-conjugated isotype specific antibody (see below) diluted in PBST. The enzyme reaction was developed by addition of 3,3',5,5'-tetramethylbenzidine (TMB; Invitrogen, Burlington, ON, Canada) and stopped after 30 min by addition of 0.5 M H_2SO_4, and the absorbance was read at 450 nm with an ELISA plate reader.

Indirect ELISA for antibodies against type 2 S. suis CPS

To perform the titration of anti-CPS mouse Ig isotypes and subclasses, 200 ng of native *S. suis* serotype 2 CPS in 0.1 M $NaCO_3$ (pH 9.6) were added to wells of an ELISA plate. After overnight coating at 4°C, plates were washed with PBST and blocked with PBS containing 1% (wt/vol) BSA for 1 h. After washing, culture supernatants or purified mAbs samples were serially diluted (2-fold) in PBST and were added to the wells and left for 1 h. After washing, antibodies were detected by incubating plates for 1 h with either HRP-conjugated goat anti-IgG plus IgM (H + L; Jackson Immunoresearch, West Grove, PA), goat

anti-IgG (Fcγ fragment specific; Jackson Immunoresearch), goat anti-IgM, goat anti-IgG1, goat anti-IgG2a, goat anti-IgG2b, or goat anti-IgG3 (Southern Biotech). The enzyme reaction was developed and read as described above. The reciprocal of the last serum dilution that resulted in an A_{450} equal to 0.1 (as a preestablished cutoff for comparison purposes) was considered the titer of that serum.

Avidity measurement

The average avidity of mAbs was determined essentially as described previously (59). Briefly, mAb dilutions producing absorbance readings at the top of the titration curve were incubated in duplicate wells coated with *S. suis* type 2 CPS (see above). Wells were then incubated for 15 min at room temperature with increasing concentrations (0 to 1 M) of the chaotrope, sodium thiocyanate (NaSCN; Sigma-Aldrich). Bound CPS is not released by treatment with NaSCN (data not shown). After incubation, NaSCN and eluted Ig were removed by extensive washings, and the wells were then incubated with HRP-conjugated goat anti-mouse IgM or IgG for 1 h (see above). The enzymatic reaction was developed until the A_{450} in the wells without NaSCN reached a value of 1.0. Since the avidity of the interaction is proportional to the resistance to elution by the chaotrope (60), avidities were expressed as avidity index (AI) (i.e., the millimolar concentration of NaSCN eluting 50% of CPS-specific mAbs). Calculations were performed as described by Goldblatt (61).

Dot-blot

Dot-blot analyses were performed as described previously by Van Calsteren *et al.* (14). Ten microliters of either heat-killed bacteria (at 1 x 10^9 CFU/ml in PBS) or purified CPS (at 1 mg/ml in 50 mM NH_4HCO_3) for serotypes 1, 2, 1/2, 14, and 9 were blotted on a PVDF Western blot membrane (EMD Millipore). The membrane was blocked for 1 h with a solution of Tris-buffered saline (TBS) containing 2% casein, followed by 2 h of incubation with mAb culture supernatants whose titers ranged from 48 to 88, with an average (± SD) titer of 65.5 ± 16.9, as determined by anti-CPS indirect ELISA. The membrane was washed, and specific HRP-conjugated anti-mouse IgM or IgG (see above) was added for 1 h. The membrane was washed three times with TBS and revealed with a 4-chloro-1-naphthol solution (Sigma-Aldrich). Densitometric analysis of dot-blot scans was performed using ImageJ version 1.49

(62). Raw integration values were normalized using respective homologous signal values with heat-killed bacteria and CPS (i.e., serotype 2 is defined as 1).

Opsonophagocytosis assay (OPA)

Murine whole blood OPAs were performed as previously described (11, 29), with some modifications. Blood was collected by intracardiac puncture from naive CD-1 mice (Charles River, Wilmington, MA), treated with sodium heparin (Sigma-Aldrich), and then diluted to obtain 6.25 x 10^6 leukocytes/ml in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, and 50 mM 2-mercaptoethanol. All reagents were from Gibco (Invitrogen). All blood preparations were kept at room temperature. Using washed bacterial cultures grown as described above, final bacterial suspensions were prepared in complete cell culture medium to obtain a concentration of 1.25 x 10^6 CFU/ml. The number of CFU/ml in the final suspension was determined by plating samples as described above. All bacterial preparations were kept on ice.

To evaluate the opsonizing capacity of the mAbs, samples were prepared by diluting to the desired concentration (indicated in the figure legends) the purified mAbs or an IgM isotype control (clone MM-30; Biolegend, San Diego, CA) in complete cell culture medium to 80 µl (40% vol/vol). To the diluted mAb samples were added 80 µl of whole blood (5 x 10^5 leukocytes) and 40 µl of either the *S. suis* serotype 2, 1, 1/2, 14 or 9 strain (5 x 10^4 CFU; multiplicity of infection [MOI] = 0.1) in a microtube to a final volume of 0.2 ml. A tube with complete cell culture medium (no antibody), whole blood and bacteria was similarly prepared as a negative control to determine the bacterial killing percentage for the mAbs. The tube tops were pierced using a sterile 25-gauge needle, and then the microtubes were incubated for 2 h at 37°C with 5% CO_2, with gentle manual agitation every 20 min. After incubation, viable bacterial counts were performed on THA using an Autoplate 4000 automated spiral plater. Also, tubes with addition of naive rabbit serum or serotype-specific rabbit anti-*S. suis* serum (63) were used as internal controls. The bacterial killing percentage was determined using the following formula: percentage of bacteria killed = [1 - (bacteria recovered from sample tubes/bacteria recovered from respective negative-control tubes)] x 100.

Bacterial agglutination assay

To evaluate the ability of the purified mAbs to agglutinate *S. suis* serotype 2, the OPA assay (see above) was adapted by replacing the 80 µl of whole blood with 80 µl of complete culture medium. The tubes contained 125 µg/ml of purified mAb and 5 x 10^4 CFU in a final volume of 0.2 ml. A tube with complete cell culture medium (no antibody) and bacteria was similarly prepared as a negative control for agglutination. The tube tops were pierced using a sterile 25-gauge needle, and then the microtubes were incubated for 2 h at 37°C with 5% CO_2, with gentle manual agitation every 20 min. After incubation, viable bacterial counts were performed as described above. Also, tubes with addition of rabbit serum were used as internal controls (see above).

Phagocytosis assay by confocal microscopy

Phagocytosis assay was adapted from (64). J774A.1 murine macrophages (ATCC TIB-67; Rockville, MD) were maintained in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 100 U/ml penicillin/streptomycin (Gibco), and cells grown at 37 °C with 5% CO_2. Confluent cell cultures were scraped, washed twice with PBS, suspended in antibiotic-free medium at 5 x 10^5 cells/ml, and incubated for 3 h at 37 °C with 5% CO_2 to allow cell adhesion onto 13 mm glass coverslips. Prior to the phagocytosis assay, to 80 µl of *S. suis* serotype 2 (5 x 10^5 CFU; MOI = 1) suspension was added either 40 µl of control rabbit serum (see above) or purified mouse mAbs (final concentration of 125 µg/ml) and 80 µl (40%) of fresh naive mouse serum as a source of complement (final volume = 0.2 ml). Bacteria were opsonized for 30 min at 37°C with end-over-end agitation. Then, pre-opsonized bacteria were added to macrophages and incubated for 1 h at 37 °C with 5% CO_2 to allow optimal phagocytosis, determined during preliminary studies (data not shown).

Coverslips were washed with PBS three times to remove non-associated bacteria. Cells were fixed and permeabilized with methanol/acetone (80:20) for 20 min at -20°C. Cells were then washed and blocked for 10 min. Coverslips were incubated for 1 h with rabbit anti-*S. suis* serotype 2 serum. After washing, coverslips were incubated with Alexa-Fluor 647 (far-red) conjugated rat anti-mouse lysosomal-associated membrane protein-1 (LAMP-1) mAb (clone

1D4B, Biolegend) and with Alexa-Fluor 488 (green) conjugated goat anti-rabbit IgG (Invitrogen) for 1 h. Staining specificity was validated using appropriate controls (Fig. S1). After washing, coverslips were incubated with DAPI for 5 min (blue; to stain the nuclei), washed and mounted on glass slides with mowiol containing DABCO. Samples were observed with an Olympus FluoView™ FV1000 confocal laser scanning microscope. Confocal microscopy images were obtained by scanning 3 times the image plane (x/y 63.121 µm; 0.079 µm/pixel), and analyzed using Fluoview software (Markham, ON, Canada).

Passive immunization and protection assay of mice

The protective capacity of the mAbs was tested in a standardized mouse model of infection using the virulent serotype 2 strain P1/7 as challenge strain (65, 66). The assay was repeated in two independent experiments (n = 10 each). Prior to the challenge, six-week-old male and female CD-1 mice (Charles River) were given a 0.2 ml intraperitoneal injection containing either the purified mAbs (at a dose of either 20 or 200 µg in PBS) or a type 2 specific rabbit antiserum prepared as described (63) (positive control; dilution 1/2 in PBS). Negative controls used were either PBS alone or an IgM isotype control (clone MM-30) at the highest dose (200 µg). One hour after passive immunization, mice were challenged with a LD90 dose of 1 x 10^8 CFU of the virulent type 2 strain P1/7 by intraperitoneal inoculation. Mice were monitored at least three times daily until 72 h p.i., and at least twice daily thereafter until the end of the study (7 days p.i.) for clinical signs and mortality. Blood bacterial burden was assessed in surviving mice 24 h and 48 h p.i. by collecting 5 µl of blood from the caudal vein. Proper dilutions were plated as described above.

Statistical analyses

Parametric data are expressed as means ± standard errors of the means (SEM) and were analyzed for significance using one way analysis of variance (ANOVA) followed by the Tukey test. Non-parametric data are shown with the median and were analyzed for significance using the Kruskall-Wallis ANOVA on ranks followed by the Dunn procedure. Statistical analyses were performed using Systat SigmaPlot version 11.0, except for the survival curves analyses, which were performed using the log-rank (Mantel-Cox) test from GraphPad Prism version 6.01. A $P < 0.05$ was considered as statistically significant.

Acknowledgements

We thank Frédéric Berthiaume for his technical assistance. This work was mainly supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a grant to MS (no. 342150) and to MG (no. 04435). Partial support was provided by Canadian Glycomics Network, funded by Networks of Centres of Excellence of Canada (grant TP-6 to MS, MG and RR) and Fonds de recherche du Québec—Nature et technologies (FRQNT) grant (no. 2018-PR-206110) to MS, MG, and RR. G. Goyette-Desjardins and J.-P. Auger are both recipients of a FRQNT Doctoral Scholarships and of an Alexander Graham Bell Graduate Scholarships—Doctoral Program from NSERC.

References

1. Gottschalk M, Xu J, Calzas C, Segura M. *Streptococcus suis*: a new emerging or an old neglected zoonotic pathogen? Future Microbiol. 2010;5(3):371-91.
2. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect. 2014;3(6):e45.
3. Segura M. *Streptococcus suis* vaccines: candidate antigens and progress. Expert Rev Vaccines. 2015;14(12):1587-608.
4. Segura M. Fisher scientific award lecture - The capsular polysaccharides of Group B *Streptococcus* and *Streptococcus suis* differently modulate bacterial interactions with dendritic cells. Can J Microbiol. 2012;58(3):249-60.
5. Roy D, Auger JP, Segura M, Fittipaldi N, Takamatsu D, Okura M, et al. Role of the capsular polysaccharide as a virulence factor for *Streptococcus suis* serotype 14. Can J Vet Res. 2015;79(2):141-6.
6. Bottomley MJ, Serruto D, Sáfadi MAP, Klugman KP. Future challenges in the elimination of bacterial meningitis. Vaccine. 2012;30(Supplement 2):B78-B86.
7. Song JY, Moseley MA, Burton RL, Nahm MH. Pneumococcal vaccine and opsonic pneumococcal antibody. J Infect Chemother. 2013;19(3):412-25.
8. Mond JJ, Kokai-Kun JF. The multifunctional role of antibodies in the protective response to bacterial T cell-independent antigens. In: Manser T, editor. Specialization and complementation of humoral immune responses to infection. Current Topics in Microbiology and Immunology. 319: Springer Berlin Heidelberg; 2008. p. 17-40.
9. Zimmermann S, Lepenies B. Glycans as vaccine antigens and adjuvants: immunological considerations. Methods Mol Biol. 2015;1331:11-26.
10. Roy R, Shiao TC. Organic chemistry and immunochemical strategies in the design of potent carbohydrate-based vaccines. Chimia (Aarau). 2011;65(1-2):24-9.

11. Goyette-Desjardins G, Calzas C, Shiao TC, Neubauer A, Kempker J, Roy R, et al. Protection against *Streptococcus suis* serotype 2 infection using a capsular polysaccharide glycoconjugate vaccine. Infect Immun. 2016;84(7):2059-75.
12. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. Biochem Cell Biol. 2010;88(3):513-25.
13. Van Calsteren MR, Gagnon F, Calzas C, Goyette-Desjardins G, Okura M, Takamatsu D, et al. Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide. Biochem Cell Biol. 2013;91(2):49-58.
14. Van Calsteren MR, Goyette-Desjardins G, Gagnon F, Okura M, Takamatsu D, Roy R, et al. Explaining the serological characteristics of *Streptococcus suis* serotypes 1 and 1/2 from their capsular polysaccharide structure and biosynthesis. J Biol Chem. 2016;291(16):8387-98.
15. Vinogradov E, Goyette-Desjardins G, Okura M, Takamatsu D, Gottschalk M, Segura M. Structure determination of *Streptococcus suis* serotype 9 capsular polysaccharide and assignment of functions of the cps locus genes involved in its biosynthesis. Carbohydr Res. 2016;433:25-30.
16. Goyette-Desjardins G, Vinogradov E, Okura M, Takamatsu D, Gottschalk M, Segura M. *Streptococcus suis* serotype 3 and serotype 18 capsular polysaccharides contain di-N-acetyl-bacillosamine. Carbohydr Res. 2018;466:18-29.
17. Okura M, Takamatsu D, Maruyama F, Nozawa T, Nakagawa I, Osaki M, et al. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: potential mechanisms for generation of capsular variation. Appl Environ Microbiol. 2013;79(8):2796-806.
18. Athey TB, Teatero S, Lacouture S, Takamatsu D, Gottschalk M, Fittipaldi N. Determining *Streptococcus suis* serotype from short-read whole-genome sequencing data. BMC Microbiol. 2016;16(1):162.
19. Roy D, Athey TBT, Auger J-P, Goyette-Desjardins G, Van Calsteren M-R, Takamatsu D, et al. A single amino acid polymorphism in the glycosyltransferase CpsK defines four *Streptococcus suis* serotypes. Sci Rep. 2017;7(1):4066.
20. Charland N, Jacques M, Lacouture S, Gottschalk M. Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by *Streptococcus suis* serotypes 1, 2 and 1/2. Microbiology. 1997;143(11):3607-14.
21. del Campo Sepulveda EM, Altman E, Kobisch M, D'Allaire S, Gottschalk M. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect ELISA. Vet Microbiol. 1996;52(1-2):113-25.
22. Calzas C, Lemire P, Auray G, Gerdts V, Gottschalk M, Segura M. Antibody response specific to the capsular polysaccharide is impaired in *Streptococcus suis* serotype 2-infected animals. Infect Immun. 2015;83(1):441-53.
23. Calzas C, Taillardet M, Fourati I, Roy D, Gottschalk M, Soudeyns H, et al. Evaluation of the immunomodulatory properties of *Streptococcus suis* and Group B *Streptococcus* capsular polysaccharides on the humoral response. Pathogens. 2017;6(2):16.
24. Gottschalk M, Higgins R, Jacques M, Mittal KR, Henrichsen J. Description of 14 new capsular types of *Streptococcus suis*. J Clin Microbiol. 1989;27(12):2633-6.
25. Wibroe PP, Helvig SY, Moein Moghimi S. The role of complement in antibody therapy for infectious diseases. Microbiol Spectr. 2014;2(2).

26. Underhill DM, Ozinsky A. Phagocytosis of microbes: complexity in action. Annu Rev Immunol. 2002;20(1):825-52.
27. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol. 2010;11(9):785-97.
28. Guilliams M, Bruhns P, Saeys Y, Hammad H, Lambrecht BN. The function of Fc receptors in dendritic cells and macrophages. Nat Rev Immunol. 2014;14(2):94-108.
29. Goyette-Desjardins G, Roy R, Segura M. Murine whole-blood opsonophagocytosis assay to evaluate protection by antibodies raised against encapsulated extracellular bacteria. In: Lepenies B, editor. Carbohydrate-Based Vaccines - Methods and Protocols. Methods in Molecular Biology. 1331. New York, USA: Springer; 2015. p. 81-92.
30. Racine R, Winslow GM. IgM in microbial infections: Taken for granted? Immunol Lett. 2009;125(2):79-85.
31. Fabrizio K, Manix C, Guimaraeš AJ, Nosanchuk JD, Pirofski L-a. Aggregation of *Streptococcus pneumoniae* by a pneumococcal capsular polysaccharide-specific human monoclonal IgM correlates with antibody efficacy in vivo. Clin Vaccine Immunol. 2010;17(5):713-21.
32. Sun Y, Hwang Y-i, Nahm MH. Avidity, potency, and cross-reactivity of monoclonal antibodies to pneumococcal capsular polysaccharide serotype 6B. Infect Immun. 2001;69(1):336-44.
33. Tian H, Weber S, Thorkildson P, Kozel TR, Pirofski LA. Efficacy of opsonic and nonopsonic serotype 3 pneumococcal capsular polysaccharide-specific monoclonal antibodies against intranasal challenge with *Streptococcus pneumoniae* in mice. Infect Immun. 2009;77(4):1502-13.
34. Elliott SD, Clifton-Hadley F, Tai J. Streptococcal infection in young pigs. V. An immunogenic polysaccharide from *Streptococcus suis* type 2 with particular reference to vaccination against streptococcal meningitis in pigs. J Hyg (Lond). 1980;85(2):275-85.
35. Wisselink HJ, Stockhofe-Zurwieden N, Hilgers LAT, Smith HE. Assessment of protective efficacy of live and killed vaccines based on a non-encapsulated mutant of *Streptococcus suis* serotype 2. Vet Microbiol. 2002;84(1–2):155-68.
36. Fabbrini M, Rigat F, Rinaudo CD, Passalaqua I, Khacheh S, Creti R, et al. The protective value of maternal Group B *Streptococcus* antibodies: quantitative and functional analysis of naturally acquired responses to capsular polysaccharides and pilus proteins in european maternal sera. Clin Infect Dis. 2016;63(6):746-53.
37. Schütz K, Hughes R, Parker A, Quinti I, Thon V, Cavaliere M, et al. Kinetics of IgM and IgA antibody response to 23-valent pneumococcal polysaccharide vaccination in healthy subjects. J Clin Immunol. 2013;33(1):288-96.
38. Weeratna RD, McCluskie MJ, Xu Y, Davis HL. CpG DNA induces stronger immune responses with less toxicity than other adjuvants. Vaccine. 2000;18(17):1755-62.
39. Berhanu A, Wilson RL, Kirkwood-Watts DL, King DS, Warren TK, Lund SA, et al. Vaccination of BALB/c mice with *Escherichia coli*-expressed vaccinia virus proteins A27L, B5R, and D8L protects mice from lethal vaccinia virus challenge. J Virol. 2008;82(7):3517-29.
40. Martelet L, Lacouture S, Goyette-Desjardins G, Beauchamp G, Surprenant C, Gottschalk M, et al. Porcine dendritic cells as an in vitro model to assess the immunological behaviour of *Streptococcus suis* subunit vaccine formulations and the polarizing effect of adjuvants. Pathogens. 2017;6(1).

41. Collins AM. IgG subclass co-expression brings harmony to the quartet model of murine IgG function. Immunol Cell Biol. 2016;94(10):949-54.
42. Sarvas HO, Seppala IJ, Tahtinen T, Peterfy F, Makela O. Mouse IgG antibodies have subclass associated affinity differences. Mol Immunol. 1983;20(3):239-46.
43. Michaelsen TE, Kolberg J, Aase A, Herstad TK, Høiby EA. The four mouse IgG isotypes differ extensively in bactericidal and opsonophagocytic activity when reacting with the P1.16 epitope on the outer membrane PorA protein of *Neisseria meningitidis*. Scand J Immunol. 2004;59(1):34-9.
44. Pincus SH, Moran E, Maresh G, Jennings HJ, Pritchard DG, Egan ML, et al. Fine specificity and cross-reactions of monoclonal antibodies to Group B streptococcal capsular polysaccharide type III. Vaccine. 2012;30(32):4849-58.
45. Weber S, Tian H, van Rooijen N, Pirofski LA. A serotype 3 pneumococcal capsular polysaccharide-specific monoclonal antibody requires Fcgamma receptor III and macrophages to mediate protection against pneumococcal pneumonia in mice. Infect Immun. 2012;80(4):1314-22.
46. Doyle CR, Moon JY, Daily JP, Wang T, Pirofski LA. A capsular polysaccharide-specific antibody alters *Streptococcus pneumoniae* gene expression during nasopharyngeal colonization of mice. Infect Immun. 2018;86(7).
47. Goldblatt D, Pinto Vaz ARJPM, Miller E. Antibody avidity as a surrogate marker of successful priming by *Haemophilus influenzae* type b conjugate vaccines following infant immunization. J Infect Dis. 1998;177(4):1112-5.
48. Usinger WR, Lucas AH. Avidity as a determinant of the protective efficacy of human antibodies to pneumococcal capsular polysaccharides. Infect Immun. 1999;67(5):2366-70.
49. Romero-Steiner S, Musher DM, Cetron MS, Pais LB, Groover JE, Fiore AE, et al. Reduction in functional antibody activity against *Streptococcus pneumoniae* in vaccinated elderly individuals highly correlates with decreased IgG antibody avidity. Clin Infect Dis. 1999;29(2):281-8.
50. Roche AM, Richard AL, Rahkola JT, Janoff EN, Weiser JN. Antibody blocks acquisition of bacterial colonization through agglutination. Mucosal Immunol. 2014;8:176.
51. Dalia Ankur B, Weiser Jeffrey N. Minimization of bacterial size allows for complement evasion and is overcome by the agglutinating effect of antibody. Cell Host Microbe. 2011;10(5):486-96.
52. Chang Q, Zhong Z, Lees A, Pekna M, Pirofski L. Structure-Function Relationships for Human Antibodies to Pneumococcal Capsular Polysaccharide from Transgenic Mice with Human Immunoglobulin Loci. Infect Immun. 2002;70(9):4977-86.
53. Fabrizio K, Manix C, Tian H, van Rooijen N, Pirofski L-a. The efficacy of pneumococcal capsular polysaccharide-specific antibodies to serotype 3 *Streptococcus pneumoniae* requires macrophages. Vaccine. 2010;28(47):7542-50.
54. Weinstein JR, Quan Y, Hanson JF, Colonna L, Iorga M, Honda S-i, et al. IgM-dependent phagocytosis in microglia is mediated by complement receptor 3, not Fcα/μ receptor. J Immunol. 2015;195(11):5309-17.
55. Katsumi M, Saito T, Kataoka Y, Itoh T, Kikuchi N, Hiramune T. Comparative preparation methods of sialylated capsule antigen from *Streptococcus suis* type 2 with type specific antigenicity. J Vet Med Sci. 1996;58(10):947-52.

56. Segura M, Stankova J, Gottschalk M. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect Immun. 1999;67(9):4646-54.
57. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975;256(5517):495-7.
58. Hale G. Isolation and purification of monoclonal antibodies from tissue culture supernatant. In: Shepherd P, Dean C, editors. Monoclonal antibodies: a practical approach. Oxford, United Kingdom: Oxford University Press; 2000. p. 149-80.
59. Colino J, Duke L, Arjunaraja S, Chen Q, Liu L, Lucas AH, et al. Differential idiotype utilization for the in vivo type 14 capsular polysaccharide-specific Ig responses to intact *Streptococcus pneumoniae* versus a pneumococcal conjugate vaccine. J Immunol. 2012;189(2):575-86.
60. Pullen GR, Fitzgerald MG, Hosking CS. Antibody avidity determination by ELISA using thiocyanate elution. J Immunol Methods. 1986;86(1):83-7.
61. Goldblatt D. Simple solid phase assays of avidity. In: Johnstone A, Turner MW, editors. Immunochemistry 2. Practical approach. New York, USA: IRL Press at Oxford University Press; 1997. p. 31-51.
62. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature methods. 2012;9(7):671-5.
63. Higgins R, Gottschalk M. An update on *Streptococcus suis* identification. J Vet Diagn Invest. 1990;2(3):249-52.
64. Segura M, Gottschalk M. *Streptococcus suis* interactions with the murine macrophage cell line J774: adhesion and cytotoxicity. Infect Immun. 2002;70(8):4312-22.
65. Dominguez-Punaro MC, Segura M, Plante MM, Lacouture S, Rivest S, Gottschalk M. *Streptococcus suis* serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection. J Immunol. 2007;179(3):1842-54.
66. Lecours MP, Fittipaldi N, Takamatsu D, Okura M, Segura M, Goyette-Desjardins G, et al. Sialylation of *Streptococcus suis* serotype 2 is essential for capsule expression but is not responsible for the main capsular epitope. Microbes Infect. 2012;14(11):941-50.
67. Varki A, Cummings RD, Aebi M, Packer NH, Seeberger PH, Esko JD, et al. Symbol Nomenclature for Graphical Representations of Glycans. Glycobiology. 2015;25(12):1323-4.

Tables

Table 1. Summary of the properties of mouse mAbs directed against *S. suis* serotype 2 capsular polysaccharide.

	Z3	**13C8**	**9E7**	**16H11**
Isotype / Subclass	IgM	IgM	IgM	IgG1
Avidity index (mM)	24	74	127	157
Serotype specificity	2, 1, and 1/2	2 and 14	2, 1, 1/2, and 14	2
Opsonic index (µg/ml)	2.35	27.8	4.35	Non-opsonic
Agglutination	+	-	+	-
Phagocytosis	+	+	+	ND[1]
Passive protection	+	+/-	+	-[2]
Origin	(20)	This sudy	This study	This study

[1] Not determined (ND).

[2] Data not shown.

Figures

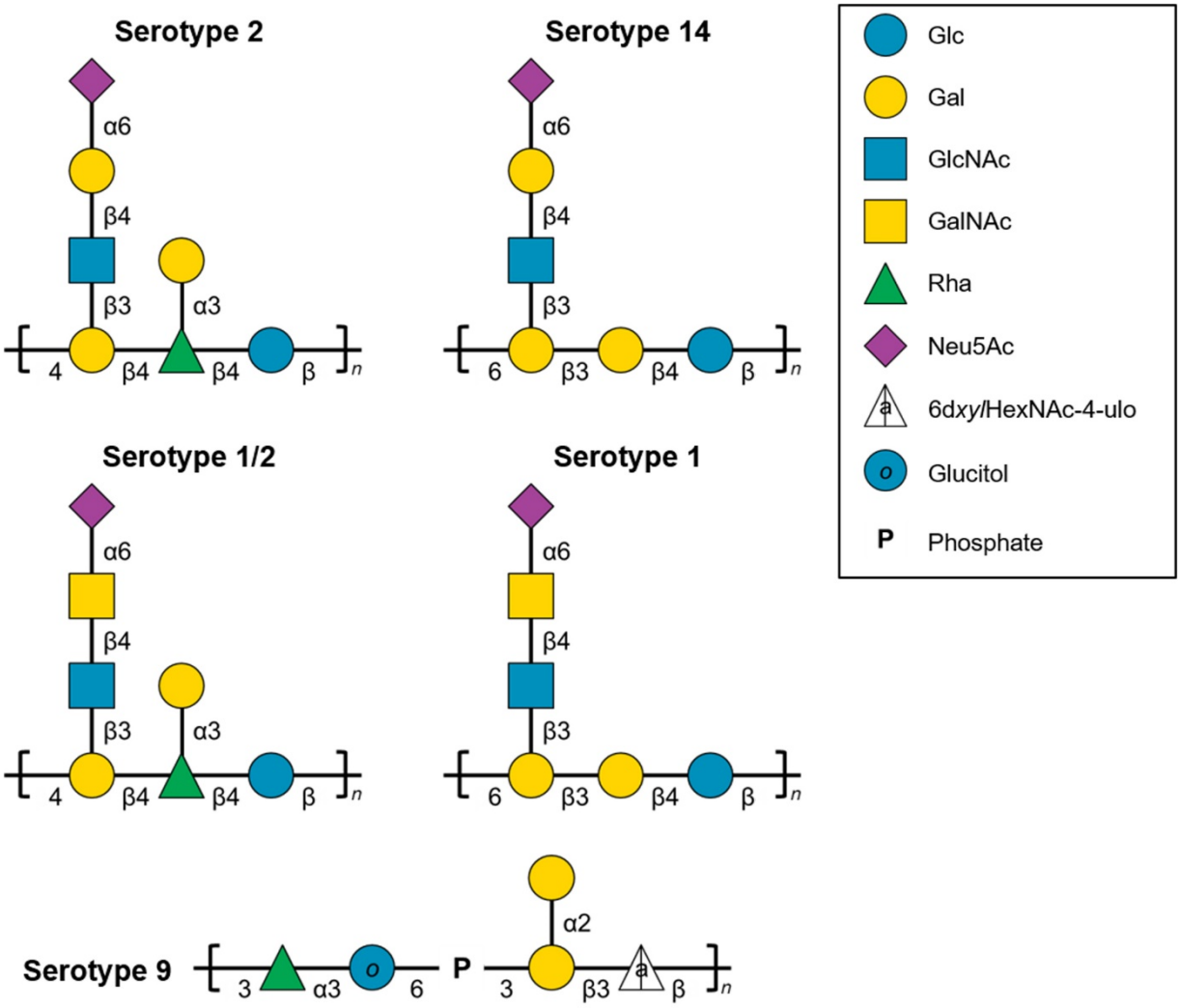


Figure 1. Comparison of reported structures for the capsular polysaccharide repeating units of *S. suis* serotypes 2 (12), 1 (14), 1/2 (14), 14 (13), and 9 (15).

Monosaccharide symbols follow the SNFG (Symbol Nomenclature for Glycans) system (67). Abbreviations: D-glucose (Glc), D-galactose (Gal), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), L-rhamnose (Rha), *N*-acetyl-D-neuraminic acid (Neu5Ac), 2-acetamido-2,6-dideoxy-β-D-*xylo*-hexopyranos-4-ulose (6d*xyl*HexNAc-4-ulo), and phosphate (P).

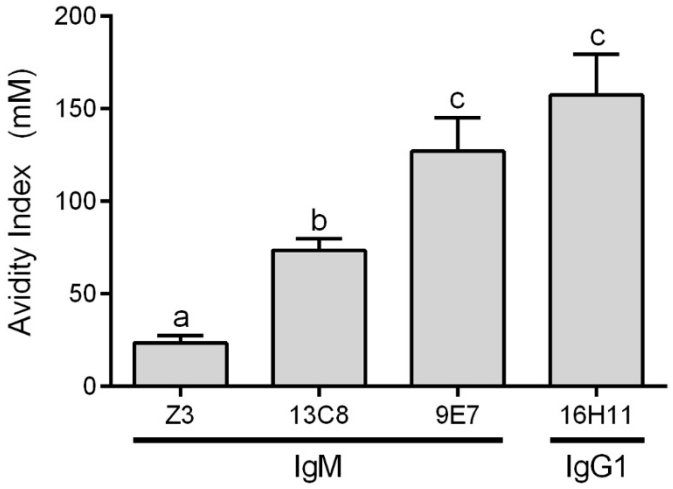


Figure 2. Avidity of mouse mAbs toward *S. suis* serotype 2 CPS.

Avidities were expressed as avidity index (i.e., the millimolar concentration of NaSCN eluting 50% of type 2 CPS-specific mAbs). mAb dilutions were chosen to produce absorbance readings at the top of the titration curve (A450 ≥ 1.0). Data are expressed as mean ± SEM of at least 3 repetitions. Significant difference between mAbs were determined by ANOVA followed by the Tukey test and is indicated by different letters ($P < 0.05$); accordingly, identical letters between groups indicate no difference.

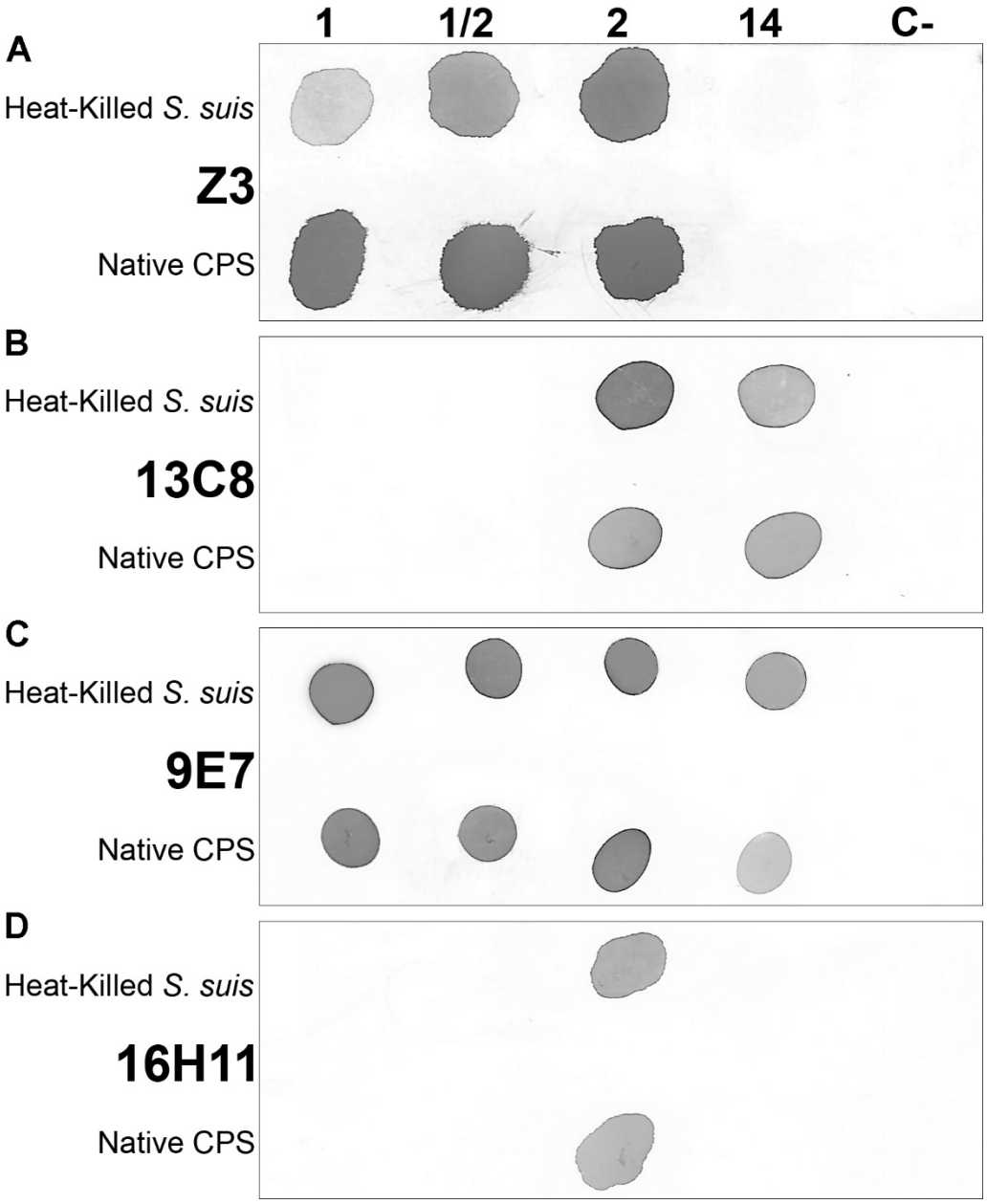


Figure 3. Serotype specificity of mAbs recognizing *S. suis* CPS.

Dot-blot analyses with 1 x 10^7 CFU of heat-killed bacteria or 10 µg of native CPS from serotypes 1, 1/2, 2, 14 or 9 (negative control) using different mouse mAb culture supernatants whose titers ranged from 48 to 88, with an average (± SD) titer of 65.5 ± 16.9, as determined by anti-CPS indirect ELISA: mAb Z3 (A), mAb 13C8 (B), mAb 9E7 (C), or mAb 16H11 (D).

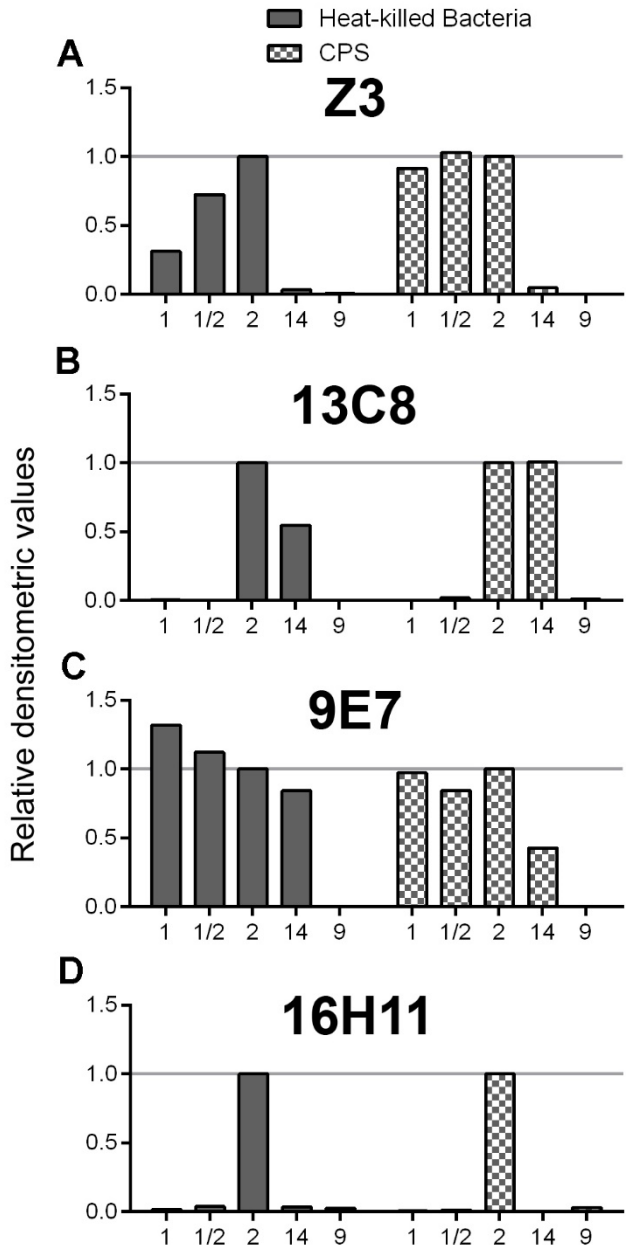


Figure 4. Densitometric analysis for the serotype specificity of mAbs recognizing *S. suis* CPS.

Integration values were obtained by densitometric analysis of each dot-blot shown in Figure 3 and are expressed relatively to the homologous reaction (i.e., serotype 2 is defined as 1) for either heat-killed bacteria or CPS: mAb Z3 (A), mAb 13C8 (B), mAb 9E7 (C), or mAb 16H11 (D).

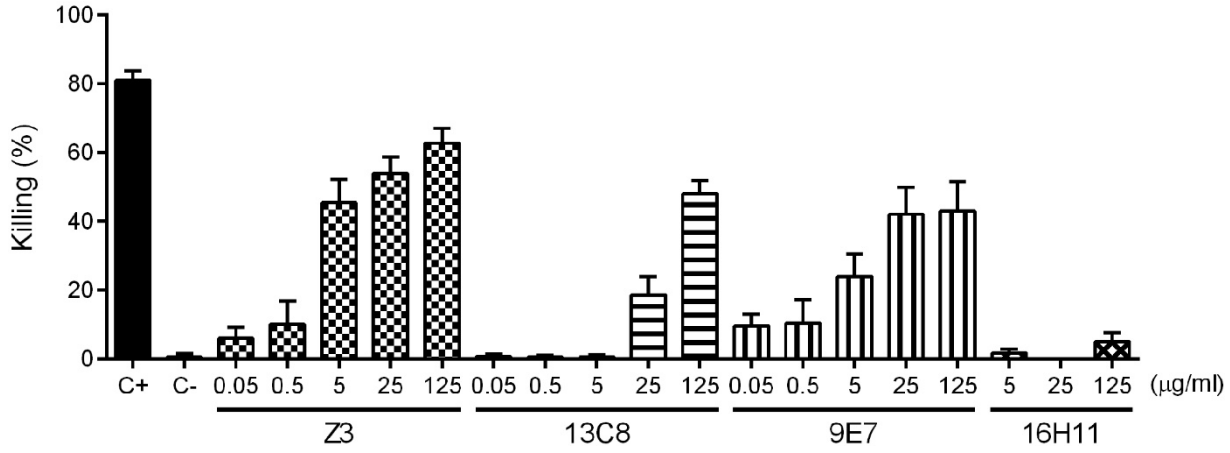


Figure 5. Opsonophagocytic killing of *S. suis* serotype 2 strain S735 by different concentrations of purified mouse mAbs.

Different concentrations (indicated on the x-axis in μg/ml) of purified mAb diluted in complete culture medium and a bacterial MOI of 0.1 were added to whole blood from naive CD-1 mice to perform the assay. Viable bacterial counts were performed after 2 h of incubation. To determine bacterial killing, viable bacterial counts from tubes incubated with mAbs were compared to those incubated without antibodies (complete culture medium + blood + bacteria). An IgM isotype control (C-) was used as a negative control for the mAbs. Serum from an hyperimmune rabbit was used as a positive control (C+) and compared to control naive rabbit serum to determine bacterial killing. Results are expressed as percent of bacterial killing for each dose, represented as mean ± SEM of at least 3 repetitions.

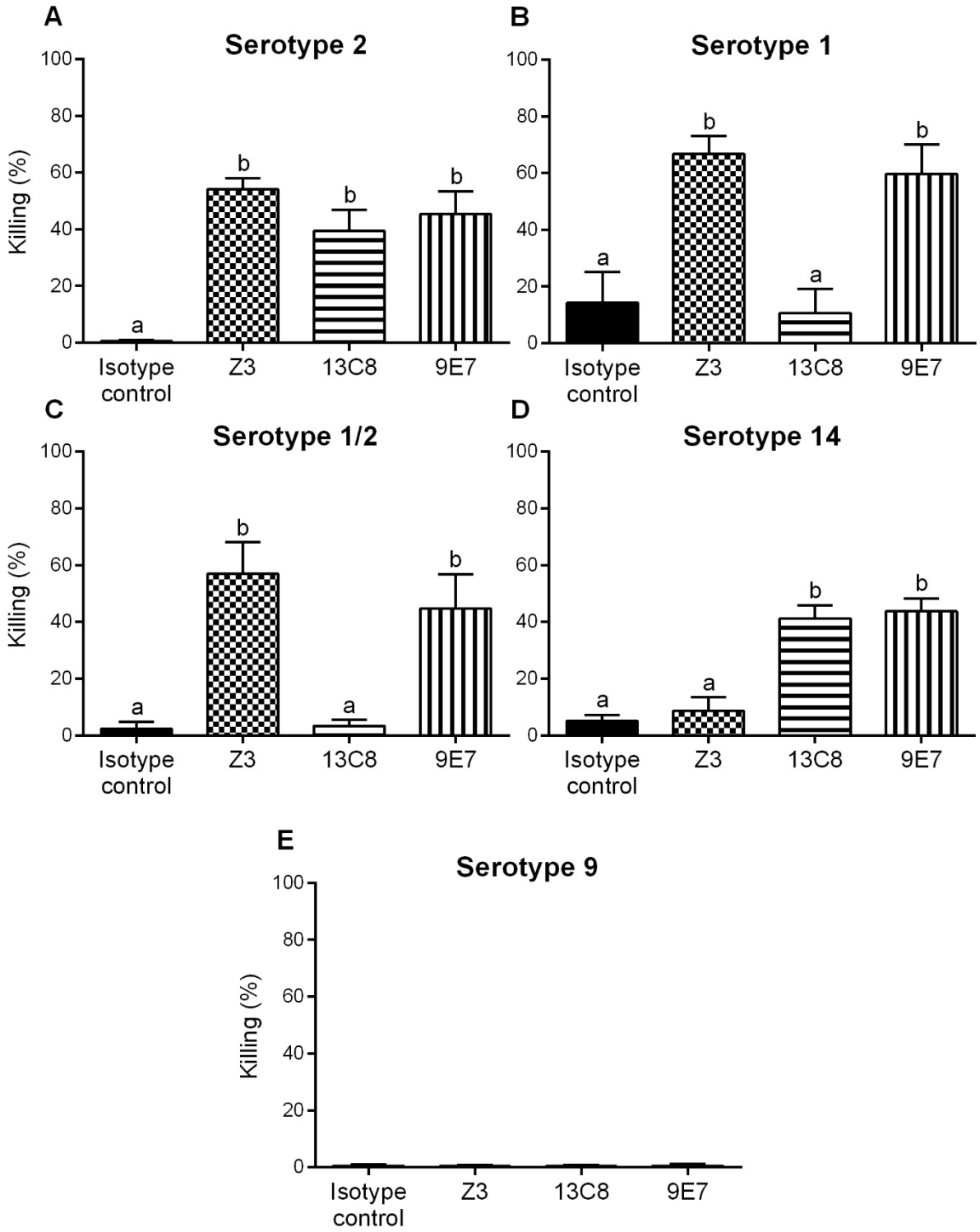


Figure 6. Opsonophagocytic killing of different *S. suis* serotypes by purified mouse mAbs.

Purified mAbs (125 µg/ml) diluted in complete culture medium and a bacterial MOI of 0.1 were added to whole blood from naive CD-1 mice to perform the assay against *S. suis* serotype 2 strain S735 (A), serotype 1 strain 1178027 (B), serotype 1/2 strain 2651 (C), serotype 14 strain DAN13730 (D), and serotype 9 strain 1273590 (E) used as a negative control. Viable bacterial counts were performed after 2 h of incubation. To determine bacterial killing, viable bacterial counts from tubes incubated with mAbs were compared to those incubated without antibodies (complete culture medium + blood + bacteria). An IgM isotype control was used as a negative control for the mAbs. Results are expressed as percent of bacterial killing for each dose, represented as mean ± SEM of at least 3 repetitions. Significant difference between mAbs and/or isotype control were determined by ANOVA followed by the Tukey test and is indicated by different letters ($P < 0.05$).

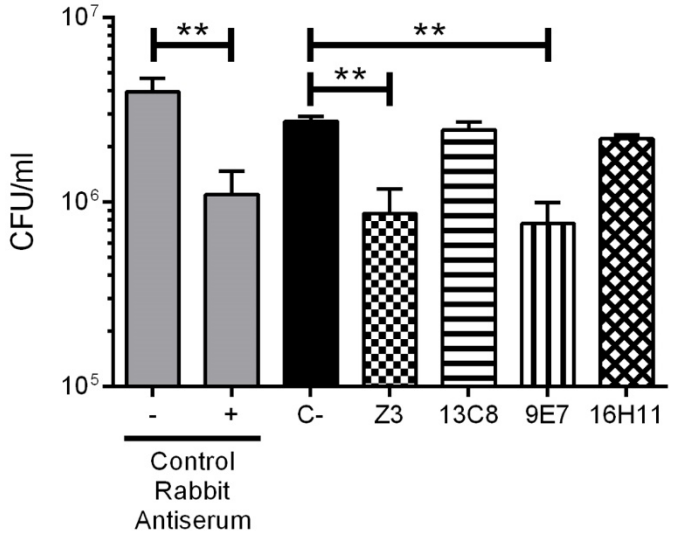


Figure 7. MAb-induced agglutination of *S. suis* serotype 2 strain S735.

Purified mAbs (125 μg/ml) diluted in complete culture medium were added to 2.5 x 10^5 CFU/ml. Viable bacterial counts were performed after 2 h of incubation and represented as mean ± SEM of at least 3 repetitions. Significant differences between mAbs and/or isotype control (C-) were determined by ANOVA followed by the Tukey test are denoted as follows: **, $P < 0.01$.

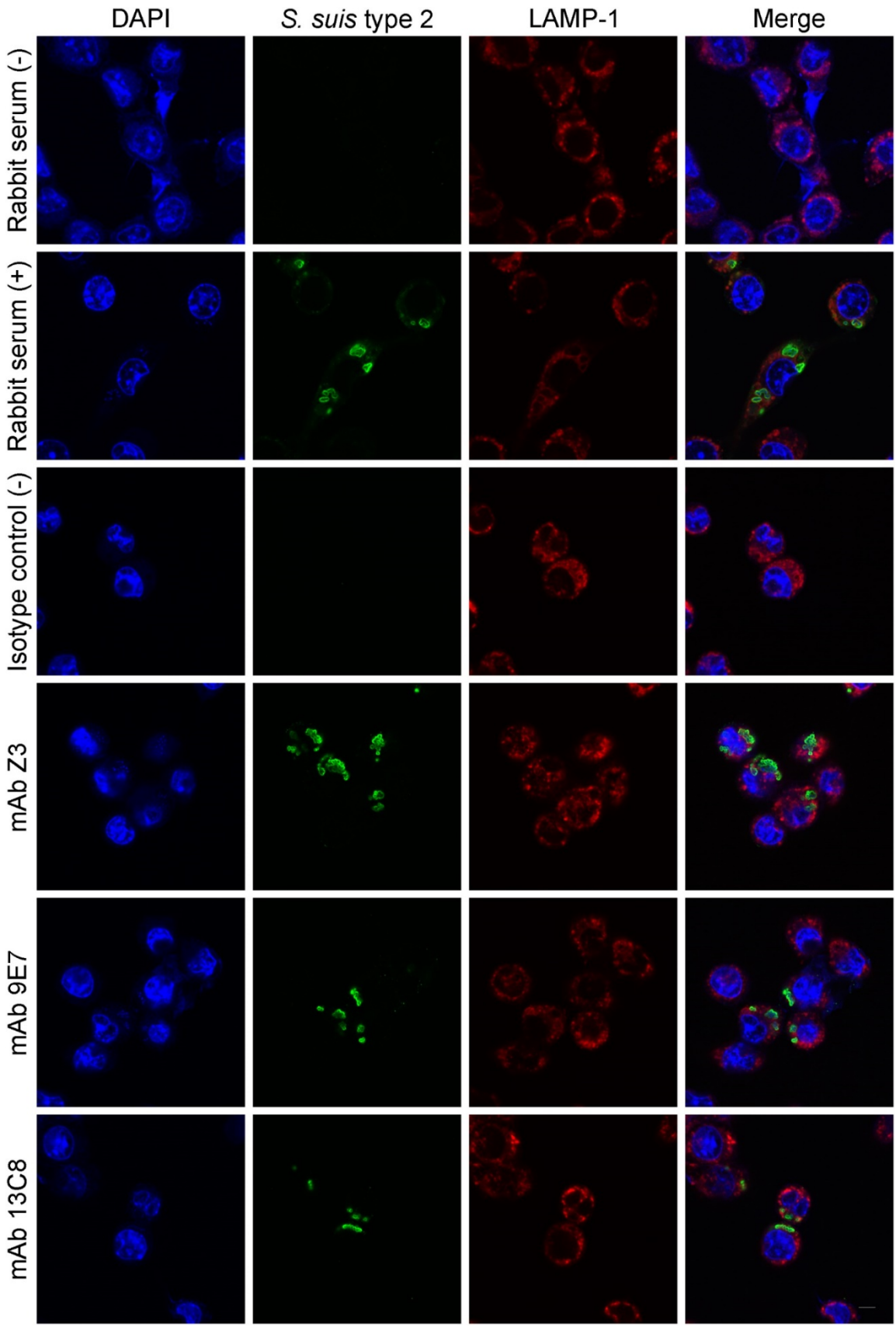

DAPI
S. suis type 2
LAMP-1
Merge
Rabbit serum (-)
Rabbit serum (+)
Isotype control (-)
mAb Z3
mAb 9E7
mAb 13C8

Figure 8. Phagocytosis of mAb-opsonized *S. suis* serotype 2 by confocal laser scanning microscopy.

After a bacteria–cell contact of 1 h, cells were fixed and labelled with serum against *S. suis* (Alexa-Fluor 488, green) and a mAb specific for LAMP-1 (Alexa-Fluor 647, red). DAPI was used to stain nuclei or bacterial DNA (blue). Merge of optical sections shows localization of opsonized *S. suis* inside LAMP-1+ vacuoles. An IgM isotype control was used as a negative control. *S. suis* opsonized with rabbit sera (either + or -) were also included as controls. Staining controls were performed and shown in Fig. S1. Scale bar = 5 μm.

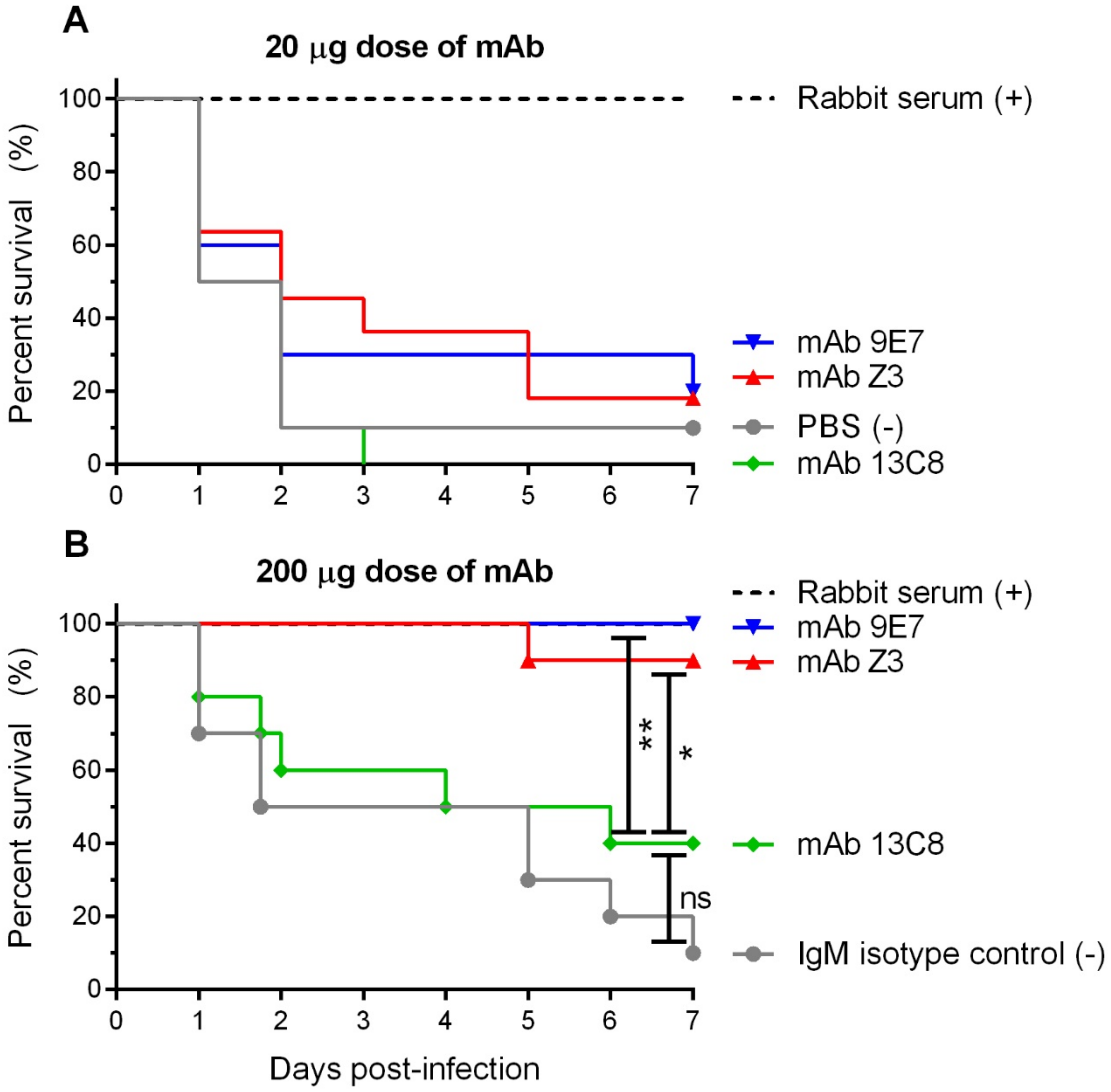


Figure 9. Passive immunization of CD-1 mice with mAbs and survival after lethal challenge with *S. suis* type 2.

Mice (n = 10) received doses of 20 μg (A, pre-trial) or 200 μg (B) of purified mAbs intraperitoneally 1 h prior to the challenge. Mice were then challenged with a LD90 dose of 1 x 10^8 CFU of *S. suis* serotype 2 strain P1/7 by intraperitoneal inoculation. Negative controls used for protection were PBS (A) or an IgM isotype control (B). Type 2 specific rabbit antiserum was used as the positive control. Significant differences between groups were determined using the log-rank (Mantel-Cox) test and are denoted as follows: not significant (ns), *, $P < 0.05$; **, $P < 0.01$.

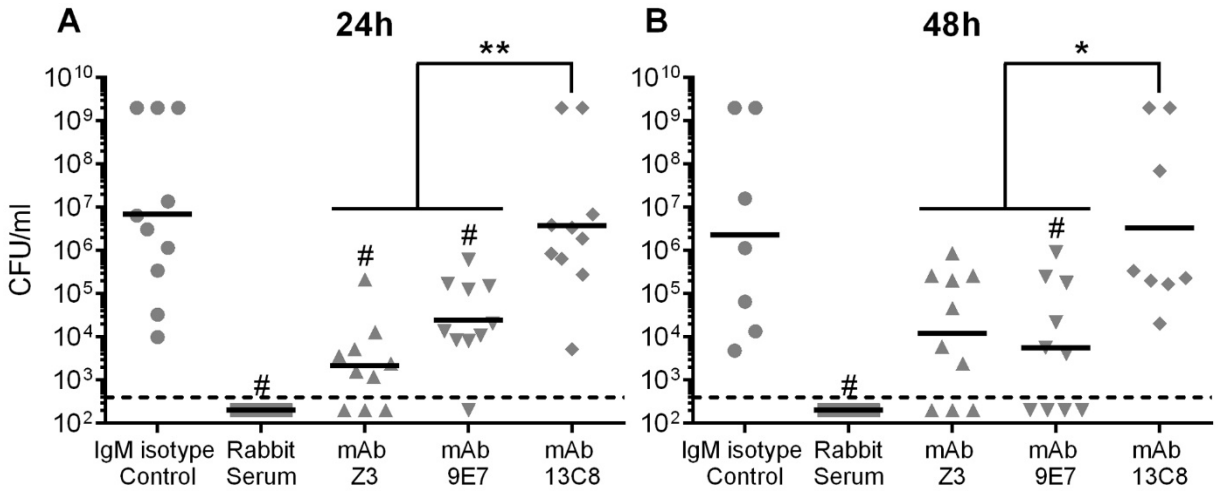


Figure 10. Passive immunization of CD-1 mice with 200 µg of mAbs and blood bacterial burden after lethal challenge.

Mice (n = 10) received a dose of 200 µg of purified mAbs intraperitoneally 1 h prior to the challenge with 1 x 10^8 CFU of *S. suis* serotype 2 strain P1/7 by intraperitoneal inoculation. Type 2 specific rabbit antiserum was used as the positive control. Bacterial counts were determined in serial dilutions of 5 µl of blood obtained from the tail vein at 24 h (A), 48 h (B), post-infection. Data of individual mice are presented as CFU/ml with the median. Significant differences between groups were determined using the Kruskall-Wallis ANOVA on ranks followed by the Dunn procedure and are denoted as follows: *, $P < 0.05$; **, $P < 0.01$. # indicates bacteremia levels significantly different from those of the isotype control group ($P < 0.05$).

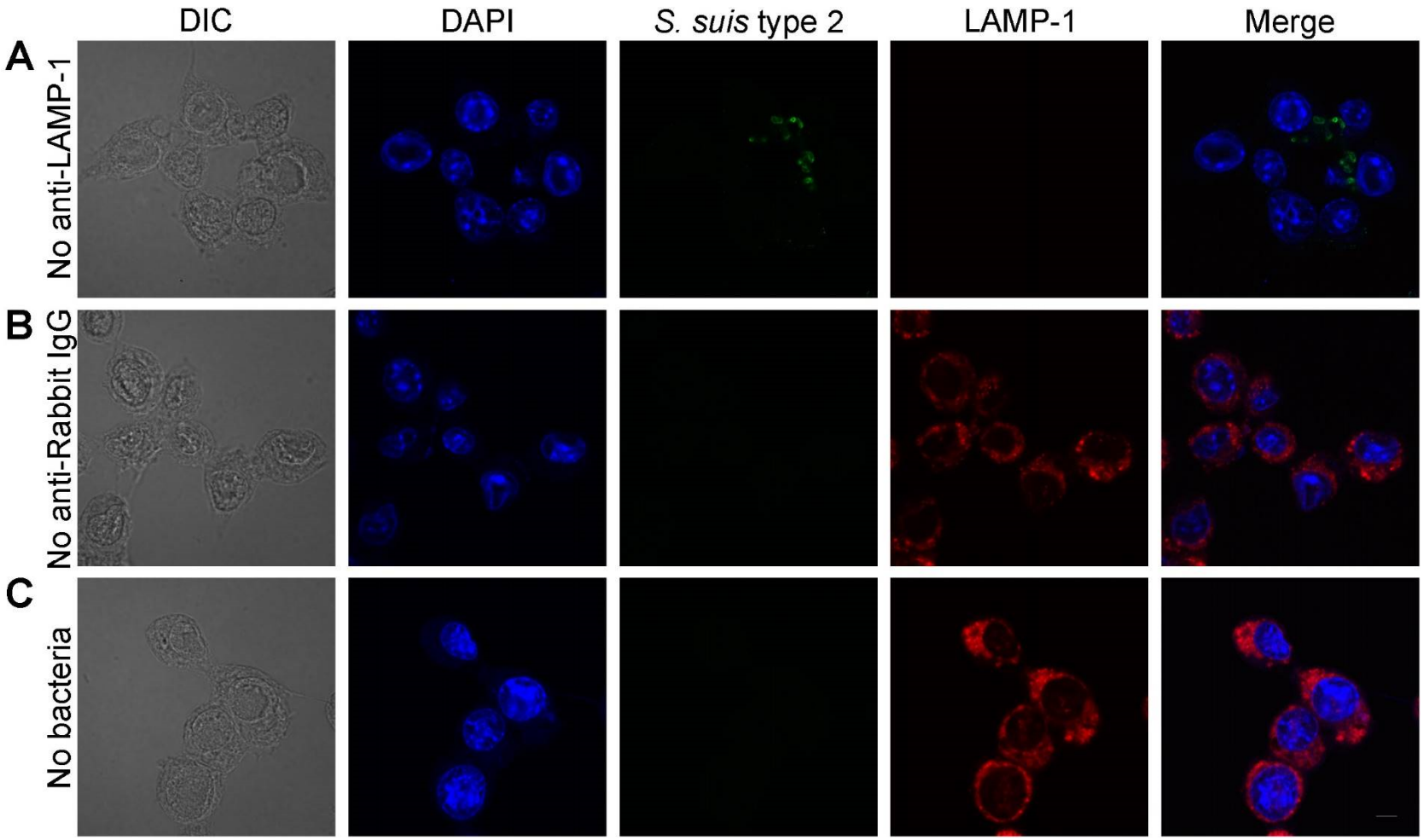

DIC
DAPI
S. suis type 2
LAMP-1
Merge
A
No anti-LAMP-1
B
No anti-Rabbit IgG
C
No bacteria

Figure S1. Staining controls for the phagocytosis of *S. suis* serotype 2 by confocal laser scanning microscopy (Fig. 8).

S. suis opsonized with positive rabbit sera (A, B) or sterile culture media (C) was added to cells. After a bacteria–cell contact of 1 h, cells were fixed and stained as follows: (A) cells were labelled with rabbit serum against *S. suis* followed by an anti-rabbit IgG (Alexa-Fluor 488, green) then stained with DAPI (blue); (B) cells were labelled with serum against *S. suis* (no conjugate) and a mAb specific for LAMP-1 (Alexa-Fluor 647, red), then stained with DAPI; (C) cells were labelled with rabbit serum against *S. suis* followed by an anti-rabbit IgG (Alexa-Fluor 488) and a mAb specific for LAMP-1 (Alexa-Fluor 647), then stained with DAPI. Scale bar = 5 μm.

IV. DISCUSSION

AXE I – Diversité des capsules polysaccharidiques de *S. suis*

1. Sérotypes importants de *S. suis* impliqués dans les infections cliniques

Avec une augmentation successive au fil des années du nombre de cas d'infections chez les humains, incluant notamment les épidémies survenues en Chine en 1998 et en 2005 avec des taux de mortalité exceptionnellement élevés, nous avons alors remarqué qu'il n'existait aucune étude récente décrivant la distribution mondiale des souches de *S. suis* impliquées dans les cas humains selon leur sérotype et leur ST, tels que déterminés par sérotypage et par MLST, respectivement. En effet, *S. suis* est bien connu pour être un pathogène hautement hétérogène, notamment grâce à ses 35 sérotypes décrits, mais d'autant plus par la grande hétérogénéité génotypique et phénotypique retrouvée parmi les souches d'un même sérotype. Également, le sérotype 2 était déjà reconnu comme étant le plus fréquemment associé aux infections chez le porc et chez l'homme (3, 252). De plus, comme le porc est l'hôte naturel de *S. suis* et que les infections chez l'homme sont toutes liées à un contact direct avec ceux-ci ou avec des produits de viande porcine, il était également essentiel de s'attarder à la distribution mondiale des souches impliquées dans les infections chez le porc. En effet, il est important de connaître la situation au niveau de la ferme afin de pouvoir pleinement apprécier la prévalence des infections chez l'homme et du potentiel zoonotique de *S. suis*.

Ainsi, les cas d'infections chez le porc (de 2002 à 2013) et chez l'homme (du premier cas en 1968 jusqu'en 2013) publiés dans la littérature ont été recensés, et l'analyse de leur distribution par sérotype et par ST a été rapportée en 2014 (**ARTICLE I**). Comme les ST n'ont pas été étudiés dans le cadre de cette thèse, ceux-ci ne seront pas discutés davantage.

1.1. Sérotypes importants chez le porc

Ainsi, au niveau mondial, nous avons pu constater que les 10 sérotypes de *S. suis* les plus fréquemment identifiés lors d'infection chez les porcs est formé, par ordre décroissant d'importance, des sérotypes 2, 3, 9, 1/2, 8, 7, 4, 22, 5 et 1 (**Figure 7**). Cependant, il est à noter que la géographie joue un rôle important sur la prévalence des sérotypes, et que ces données sont également influencées par le nombre d'études et de cas publiés.

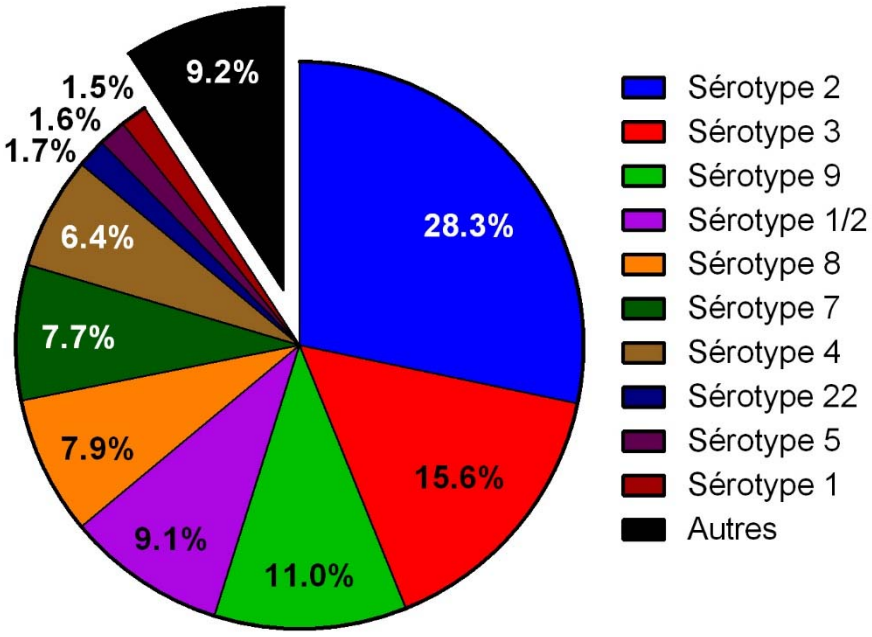


Figure 7. Prévalence des sérotypes de *S. suis* confirmés pour les cas d'infection chez les porcs de 2002 à 2013 au niveau mondial.

Préparée à partir des données de l'ARTICLE I.

Ce recensement des cas porcins a également mis en lumière un phénomène alarmant : à l'exception des données provenant du Canada, où la caractérisation de souches cliniques est rapportée de manière systématique (22, 253, 254), les données disponibles ailleurs dans le monde sont désuètes et/ou manquantes, ce qui empêche l'établissement d'un portrait précis de la situation des infections à *S. suis* au niveau des fermes porcines. Pour ce qui est de la désuétude des données, nous avons pu constater une évolution dans le temps de la prévalence de certains sérotypes, comme l'augmentation des cas de sérotypes 9 et la diminution des cas

de sérotypes 2 en Espagne (255-258). Un autre exemple d'évolution temporelle concerne la prévalence du sérotype 3 au Québec (Canada) qui a diminué continuellement de 15% [2001–2007], à 9% [2008–2011], jusqu'à 7% [2012–2014], faisant en sorte que les sérotypes dominants au Canada, par ordre décroissant d'importance, sont maintenant les sérotypes 2, 1/2 et 3 (22, 253, 254). Ensuite, pour ce qui est des données manquantes, plusieurs pays (dont plusieurs importants en production porcine) furent identifiés, avec certains notamment qui ont rapportés des cas humains alors qu'aucune donnée chez le porc n'est encore disponible ou que celles-ci sont trop désuètes.

Tel que souligné précédemment, alors que la grande majorité des cas humains d'infections à *S. suis* proviennent d'Asie, et plus particulièrement d'Asie du Sud-Est (Vietnam, Thaïlande et Chine), les données disponibles chez le porc sont très limitées, avec seulement cinq études portant sur les infections porcines provenant de Chine et de Corée du Sud. Bien que des études épidémiologiques provenant du Vietnam et de la Thaïlande existent, celles-ci se concentrent exclusivement sur des porcs en santé ou sur les carcasses des abattoirs (259-262). Le problème majeur avec les études qui se concentrent sur le portage de *S. suis* chez des animaux en santé est que celles-ci mettent en évidence (par l'échantillonnage) seulement une fraction de la population colonisant les voies respiratoires de l'animal. De plus, ces études chez les animaux en santé ne contribuent pas véritablement à brosser le portrait clinique des infections à *S. suis* : alors que la quasi-totalité des porcs sont colonisés par *S. suis*, la plupart des souches commensales sont considérées comme non virulentes. À titre d'exemple du manque de pertinence de ces études, celles-ci rapportent généralement des distributions bien différentes de celles rencontrées chez les animaux malades, avec notamment une prévalence réduite entre 3 et 14% pour le sérotype 2, comparativement à 18–57% (258-260, 263, 264). Les sérotypes 11, 14, 15, 16, 25 et 29 sont parmi les sérotypes les plus fréquemment isolés des amygdales de porcs en santé et rarement isolés lors d'infections (258-260, 263, 264).

1.2. Sérotypes importants chez l'homme

Contrairement à ce qui a été rapporté précédemment chez le porc, ce ne sont pas tous les sérotypes importants dans les infections porcines qui sont retrouvés dans les infections chez l'homme : globalement, le sérotype 2 est le plus prévalent lors d'infections chez l'homme, suivi du sérotype 14 (**Figure 8**). Pour ce qui est des infections causées par le sérotype 14, celles-ci surviennent principalement en Thaïlande (21 cas sur 33 recensés), mais également de manière plus sporadique au Vietnam (6 cas), à Taiwan (2 cas), au Canada, au Danemark, aux Pays-Bas et au Royaume-Uni (1 cas chacun). Enfin, cinq cas sporadiques causés par des sérotypes autres que 2 et 14 ont également pu être identifiés : sérotype 4 (Pays-Bas), sérotype 5 (Thaïlande), sérotype 16 (Vietnam), sérotype 21 (Argentine; **Annexes, ARTICLE VIII**) et sérotype 24 (Thaïlande).

Plus récemment, entre 2014 et 2018 ont été rapportés de nouveaux cas d'infections à *S. suis* chez les humains pour les sérotypes 1 (265), 4 (266), 5 (266-269), 9 (270) et 24 (266), en plus de 30 nouveaux cas pour le sérotype 14 en Thaïlande (266) et d'environ 800 nouveaux cas confirmés pour le sérotype 2 (266, 267, 271-284), en plus d'environ 800 cas dont le sérotype est inconnu ou incertain. Ceci amène le total des cas d'infections à *S. suis* chez l'humain rapportés à au moins 2770 depuis le premier cas de 1968.

Comme il est possible de le constater, il y a également une grande proportion (23%) des cas où le sérotype de la souche isolée demeure inconnu ou incertain (**Figure 8**). Les raisons possibles sont : premièrement, que le sérotype n'a pas été déterminé, ce qui aurait permis également de valider le diagnostic de *S. suis*; deuxièmement, que le résultat du sérotypage et/ou la méthode ayant servie à déterminer le sérotype ne sont pas indiqués dans le rapport de cas; troisièmement, que le sérotype a été identifié par des méthodes jugées inadéquates comme le sérotypage biochimique. Malgré cette grande proportion de sérotypes inconnus ou incertains, on peut supposer que la majorité de ces cas sont probablement causés par des souches de sérotype 2.

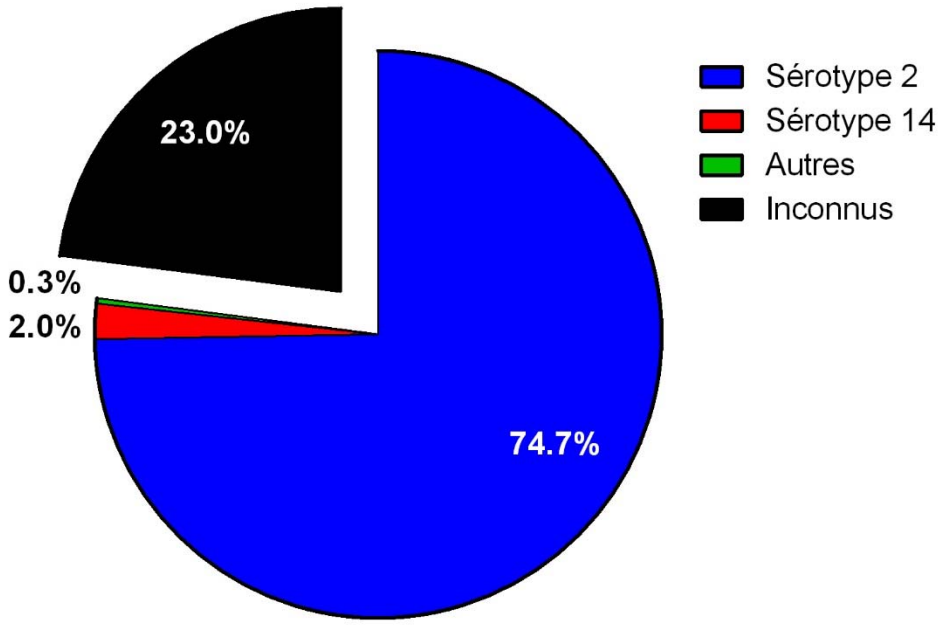


Figure 8. Prévalence des sérotypes de *S. suis* rapportés pour les cas d'infection chez les humains de 1968 à 2013 au niveau mondial.

Préparée à partir des données de l'ARTICLE I.

Une autre difficulté avec l'identification des cas humains de *S. suis* est que la majorité des laboratoires de diagnostic humains ne sont pas suffisamment sensibilisés à cet agent zoonotique et n'arrive pas à l'identifier adéquatement. De ce fait, *S. suis* peut être mal identifié comme un entérocoque, *Streptococcus pneumoniae*, *Streptococcus bovis*, un streptocoque du groupe viridans ou même *Listeria monocytogenes* (1). D'autant plus, chez les patients présentant des signes de méningite, une identification préliminaire de coques à Gram positif dans un échantillon de sang et/ou de liquide cérébro-spinal va permettre au médecin traitant de débuter un traitement aux antibiotiques, qui dans la majorité des cas va permettre d'enrayer l'infection sans devoir pousser l'identification de la souche jusqu'à l'espèce. Une bonne partie des cas humains rapportés depuis les années 2000 comme étant *S. suis* provient d'études rétrospectives où des techniques de biologie moléculaire plus sensibles (comme le séquençage de l'ARN 16S ou l'emploi de la PCR) sont employées pour valider l'identification initiale des souches. Cette problématique d'identifier correctement les souches de *S. suis*, et donc la possible sous-estimation du nombre réel de cas humains, permet donc d'expliquer, du moins

en partie, le faible nombre de cas rapportés au niveau mondial, l'exception étant les laboratoires de diagnostic humains en Asie du Sud-Est; ceux-ci sont bien informés et incluent constamment *S. suis* dans leur diagnostic différentiel, puisque *S. suis* est reconnu comme étant la première cause au Vietnam et la deuxième cause en Thaïlande de méningite chez l'adulte, en plus d'être la troisième cause plus fréquente de méningite bactérienne à Hong Kong (285-287).

Plus récemment, l'adoption de nouvelles technologies plus rapides et fiables que les tests biochimiques et les milieux chromogéniques classiques pour le diagnostic des pathogènes bactériens sont de plus en plus utilisées dans les laboratoires diagnostiques, comme l'emploi du diagnostic moléculaire (par PCR, micropuces à ADN ou séquençage du génome entier) et de méthodes protéomiques (par spectrométrie de masse) (288). L'arrivée de ces nouvelles technologies dans les laboratoires de diagnostic promet de contribuer à mieux identifier les infections causées par *S. suis* chez l'homme, et ainsi nous permettre d'obtenir un portrait plus fiable des infections chez l'homme. On peut déjà apercevoir cette tendance par l'augmentation considérable du nombre de cas humains de *S. suis* rapportés dans la littérature depuis les années 2000, se chiffrant aujourd'hui à approximativement une centaine de cas annuellement (**Figure 9**).

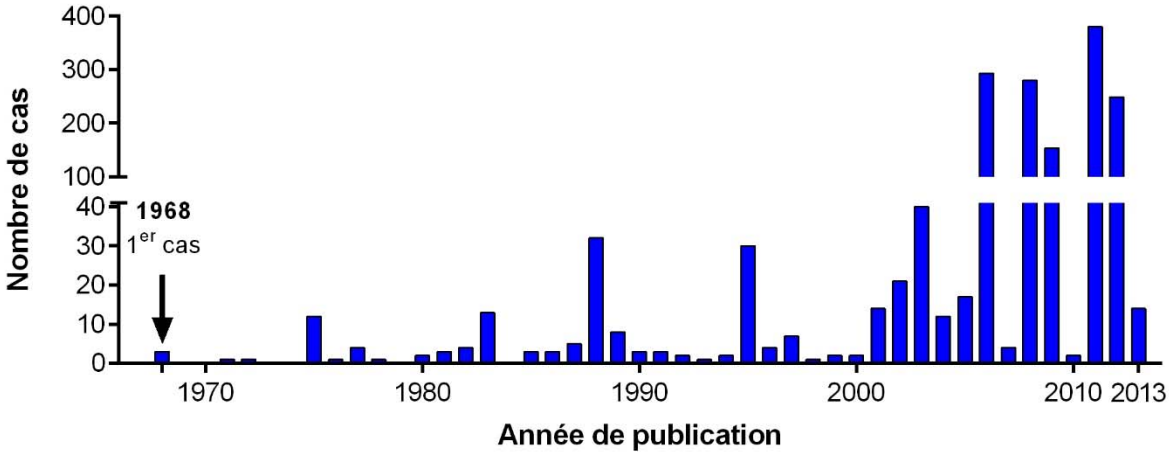


Figure 9. Nombre de cas humains d'infections à *S. suis* rapportés par année.

Préparée à partir des données de l'ARTICLE I.

Pour conclure cette première partie, malgré la limitation des données disponibles sur la caractérisation des sérotypes dans les cas d'infections par *S. suis*, un portrait global et général des sérotypes les plus importants a pu être brossé. Alors que le top 10 des sérotypes les plus importants chez le porc représentent plus de 90% des cas (spécifiquement les sérotypes 2, 3, 9, 1/2, 8, 7, 4, 22, 5 et 1), le sérotype le plus prévalent lors d'infections chez l'homme est le sérotype 2, suivi du sérotype 14. Ceci suggère que les souches virulentes chez le porc ne possèdent pas toutes le même potentiel zoonotique. Ainsi, pour la plupart des régions du monde, nous avons confirmé que le sérotype 2 de *S. suis* est toujours le sérotype le plus fréquemment isolé et le plus associé avec les infections autant chez le porc que chez l'homme.

Ces données sur la prévalence des sérotypes et de leur distribution géographique permettront d'orienter la recherche réalisée sur *S. suis*, notamment sur la pathogenèse et les facteurs de virulence, mais également sur la détermination des structures des CPS de *S. suis* et le développement de futurs vaccins efficaces basés sur les sérotypes. De plus, ce portrait épidémiologique indique plusieurs lacunes qui nécessitent d'être comblés, spécialement concernant plusieurs pays qui sont d'importants producteurs de porc.

2. Détermination des structures des CPS de *S. suis*

Compte tenu de l'importance épidémiologique considérable du **sérotype 2** de *S. suis*, autant chez le porc que chez l'humain, ce fut la première structure de CPS à être décrite en 2010 par Van Calsteren et collaborateurs (154) . Le second sérotype pour lequel la structure de la CPS a été élucidée fut le **sérotype 14** en 2013, basé sur son importance pour les cas humains d'infection à *S. suis* (**Annexes, ARTICLE IX**). Les structures des CPS de *S. suis* caractérisées jusqu'à présent sont illustrées à la **Figure 10**.

Par la suite, ce fut au tour des structures de CPS pour les **sérotypes 1 et 1/2** d'être déterminées en 2016, principalement afin de comprendre les réactions croisées rencontrées à

la sérotypie entre ces deux sérotypes et avec les sérotypes 2 et 14 de *S. suis* (**Annexes, ARTICLE X**). L'antigénicité de ces quatre sérotypes, ainsi que l'analyse des éléments structuraux susceptibles de causer ces réactions croisées, seront discutés plus tard. Ici, il s'agit de mentionner que structuralement parlant, la CPS du sérotype 2 est identique à celle du sérotype 1/2 et celle du sérotype 14 est identique à celle du sérotype 1, avec comme exception que le galactose (Gal) porteur de l'acide sialique (Neu5Ac) des sérotypes 2 et 14 est remplacé par la *N*-acétylgalactosamine (GalNAc) chez les sérotypes 1 et 1/2 (**Figure 10**). Il est également à noter que ces réactions croisées obtenues par sérotypie ne peuvent pas être résolues en sérotypant par PCR; en effet, cette substitution Gal/GalNAc a pu être associée au polymorphisme d'un seul acide aminé dans la glycosyltransférase CpsK (W161 pour les sérotypes 2 et 14; C161 pour les sérotypes 1/2 et 1) qui définit la prédilection de substrat entre le Gal et le GalNAc, qui par conséquent détermine la composition de la CPS, sa structure et le sérotype de la souche (289) (**Annexes, ARTICLE XII**).

Ensemble, les CPS des sérotypes 2, 14, 1 et 1/2 constituent une famille dont les structures sont hautement similaires, notamment car elles possèdent toutes au moins une chaîne latérale terminée par un Neu5Ac lié en α2,6- sur le Gal/GalNAc porteur, ainsi que des compositions relativement restreintes de Glc, Gal, GlcNAc et de Rha. Étant faiblement diversifiée, cette famille structurale évoque les structures des différentes CPS de GBS (**Figure 4**). En effet, les CPS de GBS possèdent toutes au moins une chaîne latérale terminée par un Neu5Ac lié en α2,3- sur le Gal porteur, avec des compositions relativement restreintes de Glc, de Gal, de GlcNAc et de Rha.

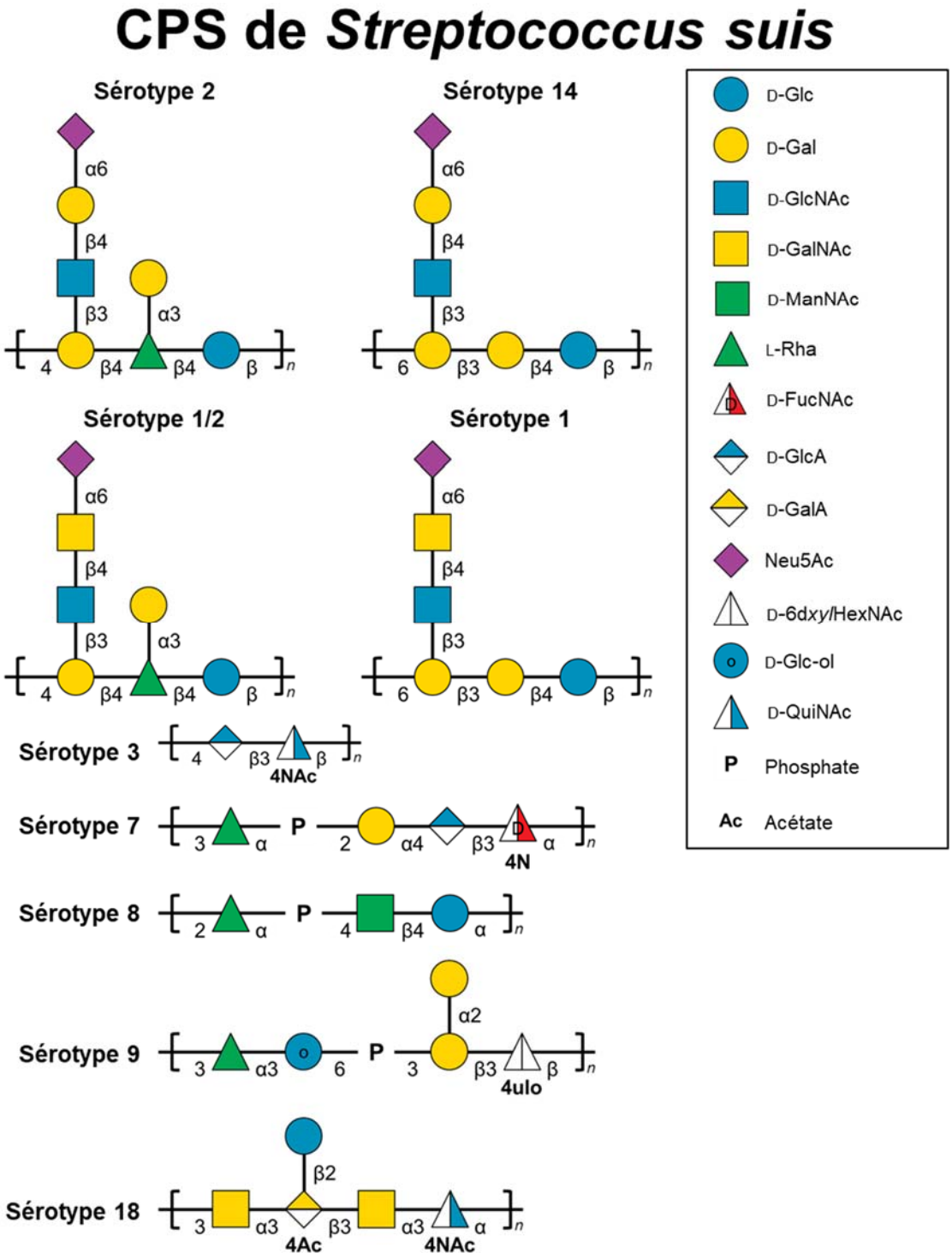


Figure 10. Structures des unités répétitives des CPS de *S. suis* qui ont été décrites jusqu'à présent.

Les symboles employés pour représenter les monosaccharides suivent le système de « Symbol Nomenclature for Glycans » tel qu'établi par Varki et collaborateurs (155). Abréviation: D-6d-*xyl*HexNAc, 2-acétamido-2,6-didésoxy-β-D-*xylo*-hexose.

Nous avons donc poursuivi la détermination des structures de CPS de *S. suis* en suivant le top 10 des sérotypes les plus importants chez le porc. Compte tenu de sa forte prévalence en Europe, la structure de la CPS du **sérotype 9** fut élucidée et rapportée en 2016 (**Annexes, ARTICLE XI**). La structure de la CPS de ce sérotype est fort différente des structures et compositions rencontrées auparavant avec les sérotypes 2, 14, 1 et 1/2 : la CPS du sérotype 9 contient notamment un sucre-6-phosphate, un polyol (soit le glucitol [Glc-ol]), une cétone labile (soit le 2-acétamido-2,6-didésoxy-D-*xylo*-hexopyranos-4-ulose, abrégé comme D-6d*xyl*HexNAc-4-ulo ou encore « Sug » (**Figure 10**). Ce fameux « 4-keto Sug » se retrouve également dans la CPS du sérotype 5 de *S. pneumoniae* (**Figure 5**).

La structure de CPS à laquelle nous nous sommes attaqués ensuite fut celle du **sérotype 3** (**ARTICLE II**). L'unité répétitive est composée d'un disaccharide formé d'acide glucuronique (GlcA) et d'un sucre rare produit seulement par certains procaryotes, soit la di-*N*-acétylbacillosamine (QuiNAc4NAc) (**Figure 10**). De plus, par analyses génétiques recherchant les enzymes responsables de la biosynthèse de la QuiNAc4NAc dans les *loci* de synthèse des CPS de *S. suis*, le **sérotype 18** fut identifié comme possédant également ce sucre rare. L'unité répétitive de la CPS du sérotype 18 contient notamment un acide galacturonique (GalA) 4-acétylé (**Figure 10**). Enfin, des possibilités intéressantes pour de futures études immunologiques des CPS des sérotypes 3 et 18 s'offrent à nous, puisqu'étant un sucre unique à certains procaryotes, il a été proposé que la QuiNAc4NAc pourrait servir de leurre pour distraire le système immunitaire (290).

Finalement, nous avons également élucidé les structures des CPS pour les **sérotypes 7 et 8** (**ARTICLE III**). Du point de vue de la composition, ces deux CPS contiennent chacune un rhamnose-1-phosphate, ce qui les rend particulièrement sensibles et nécessite des précautions particulières lors de la purification et de leur manipulation lorsqu'ils sont en solution (**Figure 10**). La CPS du sérotype 7 comprend, en plus du phosphate chargé

négativement, un GlcA (chargé négativement) et un 2-acétamido-4-amino-2,4,6-tridésoxy-D-galactose (D-FucNAc4N ou AATGal) où le groupement 4-amino est chargé positivement. Bien que la CPS du sérotype 7 porte à la fois des charges positives et négatives, sa charge globale est négative comme pour toutes les autres CPS de *S. suis* décrites jusqu'à présent. En ce qui a trait au sérotype 8, sa CPS est identique à celle du sérotype 19F de *S. pneumoniae*, et contient entre autres une *N*-acétylmannosamine (ManNAc).

Au contraire de la famille structurale formée par les CPS des sérotypes 2, 14, 1 et 1/2, les CPS des sérotypes 3, 7, 8, 9 et 18 ont été démontrées comme ayant une grande diversité compositionnelle et structurale, ce qui évoque les structures des CPS rapportées pour *S. pneumoniae* (**Figure 5**). En poursuivant les travaux de description des structures des CPS de *S. suis* ainsi que l'augmentation continue du nombre de génomes entiers bactériens assemblés disponibles (par la démocratisation du séquençage d'ADN à haut débit), il sera possible éventuellement de pouvoir réaliser des études visant à élucider l'évolution des sérotypes de *S. suis* (157, 289).

Pour conclure cette deuxième partie sur la détermination des structures de CPS, au sérotype 2 qui était la seule structure de CPS connue au début de ce projet, nous avons contribué les structures des unités répétitives pour 8 CPS de *S. suis* additionnelles, soit celles des sérotypes 1, 1/2, 3, 7, 8, 9, 14 et 18.

Ces informations sur la composition et la structure des CPSs sont essentielles, puisque celles-ci sont à la base de toute étude subséquente concernant leur antigénicité et leur immunogénicité. De ce fait, l'élucidation des structures de CPS constitue la première étape de toute démarche visant la création et l'étude de vaccins basés sur les CPS, et justifie de poursuivre l'élucidation des structures pour les 35 sérotypes de *S. suis*.

AXE II – Réponse immunitaire humorale face aux capsules polysaccharidiques de *S. suis*

3. Antigénicité et immunogénicité des CPS de *S. suis*

3.1. Éléments structuraux des CPS reconnus par les sérums employés en sérotypie

Grâce aux travaux de détermination des structures de CPS et à la disponibilité des CPS purifiées pour ces sérotypes, il a été possible pour la première fois d'étudier les réactions croisées avec les sérums utilisés pour la sérotypie par analyse de dot-blot : cette technique consiste à déposer directement sur une membrane de « polyvinylidene fluoride » (PVDF) les antigènes à l'étude (ici nos CPS purifiées), puis à réaliser l'immunoblot des échantillons en les incubant d'abord avec un sérum primaire (ici les sérums de lapin de sérotypie) puis avec un anticorps secondaire conjugué permettant de mettre en évidence après révélation la liaison de l'anticorps à son antigène. De plus, le choix du dot-blot pour réaliser ces études est approprié, puisque les réactions croisées obtenues avec les sérums de lapin contre les différents sérotypes furent identiques aux réactions obtenues à la coagglutination, méthode de référence pour la sérotypie des souches de *S. suis* (**Annexes, ARTICLE X**). Enfin, pour l'étude des CPS purifiées des sérotypes 2, 14, 1 et 1/2, qui contiennent toutes de l'acide sialique, nous avions également comme objectif d'évaluer le rôle de ce sucre dans la reconnaissance par les anticorps, compte tenu de son importance pour le sérotype III de GBS notamment (discuté plus loin).

3.1.1. Réaction croisée entre les sérotypes 1 et 14

La grande similarité structurale entre les sérotypes 14 et 1 (substitution du Gal en GalNAc) permet aisément d'expliquer leurs réactivité croisées mutuelles (**Annexes, ARTICLE X**). Cependant, la reconnaissance de la CPS du sérotype 14 (CPS 14) par le sérum anti-sérotype 1 est plus faible que la réaction homologue (c'est-à-dire de CPS 1 par le sérum

anti-sérotype 1), ce qui indique que la non-substitution du Gal affecte la reconnaissance optimale par le sérum anti-sérotype 1. Ces résultats suggèrent que le sérum anti-sérotype 1 reconnait principalement la chaîne latérale. Pour ce qui est du sérum anti-sérotype 14, celui-ci reconnait également les CPS 1 et 14, suggérant que l'épitope dominante reconnue par celui-ci est située au niveau de la chaîne principale du polysaccharide. En effet, ces résultats sont également supportés par l'absence de reconnaissance de la CPS 1/ 2 (dont la chaîne latérale est identique à celle de la CPS 1) par le sérum anti-sérotype 14. De plus, comme les versions natives et désialylées pour les sérotypes 1 et 14 et leurs réactifs de sérotypie ont produits des réactions d'intensité égale, on peut conclure que l'acide sialique terminal de ces deux CPS ne joue aucun rôle dans la reconnaissance de ces sérotypes en sérotypie. Ainsi, lors de l'identification par sérotypie de souches de *S. suis* positives pour le sérotype 14, il est important de tester également contre le sérotype 1 afin d'identifier certainement le sérotype de la souche donnée (1 ou 14).

3.1.2. Réaction croisée du sérotype 1/2 avec les sérotypes 1 et 2

Pour ce qui est de la reconnaissance du sérotype 1/2 par les antisérums contre les sérotypes 1, 1/2 et 2, tout semble se jouer au niveau de la chaîne latérale sialylée (**Annexes, ARTICLE X**). De plus, comme il a été mentionné précédemment que le sérum anti-sérotype 1 reconnait principalement la chaîne latérale, ceci permet donc d'expliquer cette réaction croisée, puisque les chaînes latérales des CPS 1 et 1/2 sont identiques (GalNAc toutes les deux). Pour ce qui est la reconnaissance par le sérum anti-sérotype 2, celui-ci reconnait plus faiblement la CPS 1/2 que sa réaction homologue (avec CPS 2); étant donné que ces deux CPS sont identiques à l'exception du GalNAc de la CPS 1/2, ceci supporte que la reconnaissance se fait majoritairement au niveau de la chaîne latérale sialylée. Ainsi, lors de l'identification par sérotypie de souches de *S. suis* positives soit pour le sérotype 1 ou 2, il est important de tester contre ces sérotypes afin d'identifier certainement le sérotype de la souche donnée (1, 2 ou 1/2). Finalement, compte tenu de l'importance de la structure de la chaîne latérale dans ces réactions avec le sérotype 1/ 2, on peut conclure que l'acide sialique joue un rôle essentiel.

3.1.3. L'énigme de l'épitope du sérotype 2

Des travaux précédents étudiant la reconnaissance des CPS native et désialylée du sérotype 2 par l'antisérum homologue ont rapporté des résultats contradictoires concernant l'importance de l'acide sialique dans cette reconnaissance (**Annexes, ARTICLES XIV et XV**). De plus, tel qu'il a été observé lors de l'étude des réactions croisées avec le sérotype 1/2 (**Annexes, ARTICLE X**), il apparait que la chaîne latérale sialylée soit reconnue par une population majoritaire d'anticorps, bien que la CPS désialylée demeure faiblement reconnue par une population minoritaire d'anticorps. Afin de clarifier cette situation, des dot-blots ont été réalisés en employant sept différents lots de sérums de lapin produits contre le sérotype 2 et employés pour la sérotypie de *S. suis* (résultats non présentés). Ces expériences ont permis de mettre en évidence que sur les sept lots de sérums testés, cinq reconnaissent plus faiblement la CPS désialylée, alors que les deux autres sont incapables de la reconnaître. Une expérience supplémentaire a été réalisée à l'aide de deux lots différents de CPS natives et désialylées et d'un lot de sérum de chaque groupe (faible ou forte dépendance au Neu5Ac) qui ont été utilisés à trois différentes dilutions (**Figure 11**). Ainsi, ces résultats démontrent l'existence d'au moins deux populations d'anticorps dans les sérums de lapin employés pour la sérotypie du sérotype 2 : une première population majoritaire/dominante qui reconnait exclusivement la CPS native sialylée, ainsi qu'une seconde population (quantité et/ou spécificité variable(s) selon le lot de sérum utilisé) minoritaire/mineure reconnaissant probablement la chaîne principale du polysaccharide. En effet, en diluant suffisamment les sérums, on peut perdre le signal déjà faible contre la CPS désialylée alors que la reconnaissance de la CPS native n'est pas affectée.

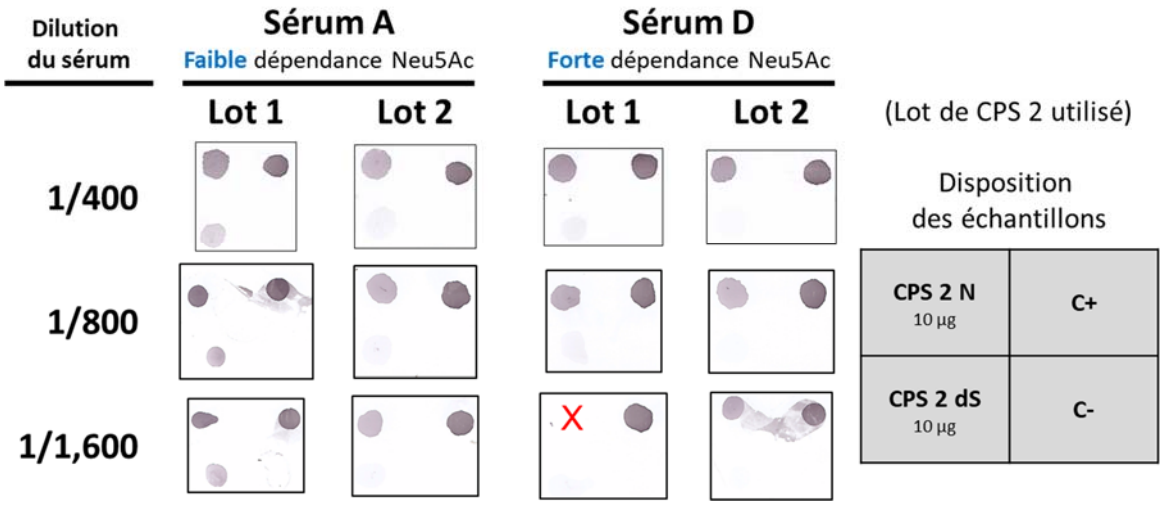


Figure 11. La chaîne latérale sialylée de la CPS du sérotype 2 est majoritairement reconnue par les sérums anti-sérotype 2.

Les dot-blots ont été réalisés tel que décrit dans (**Annexes, ARTICLE X**). Deux sérums différents de lapin (un ayant une faible dépendance à la présence du Neu5Ac et l'autre étant fortement dépendant) à trois différentes dilutions ainsi que deux différents lots de CPS natives (N) et désialylées (dS) ont été employés pour valider les différences inter-lots. Le contrôle positif (C+) utilisé est 10^7 CFU de la souche S735 de *S. suis* sérotype 2 tué à la chaleur tel que décrit dans (**ARTICLES IV, VI et VII**). Le contrôle négatif (C-) utilisé est le NH_4HCO_3 50 mM, tampon ayant servi à solubiliser les préparations de CPS. Le X rouge dénote une erreur technique survenue lors du dépôt des échantillons, ce qui explique l'absence de signal.

De plus, le fait que les anticorps dirigés contre la chaîne latérale de la CPS 1 (dans le sérum anti-sérotype 1) peuvent reconnaître la CPS 14, mais non la CPS 2 alors que leurs chaînes latérales sont identiques, suggère un contrôle structural et/ou conformationnel par le Neu5Ac et/ou encore par la chaîne du polysaccharide de la CPS 2, plus probablement par la seconde chaîne latérale formée du Gal lié en α1,3- sur le Rha (**Annexes, ARTICLE X**). Une des difficultés rencontrées avec l'hydrolyse acide douce des CPS pour éliminer l'acide sialique est que celles-ci subissent également une dépolymérisation [de 410–480 à 21–23 kDa pour CPS 2, tel que rapporté dans (154) et (**Annexes, ARTICLE XV**)]. L'hypothèse serait que cette forte dépolymérisation soit liée à la présence de Rha dans la chaîne principale du polysaccharide, ce qui a pu être confirmé avec la forte dépolymérisation de la CPS 1/2 comparativement à la faible dépolymérisation subie par les CPS 1 et 14 qui ne contiennent pas de Rha (**Annexes, ARTICLES IX, X et XV**). Afin de pouvoir dissocier l'effet de la longueur de la chaîne de l'effet de la présence d'acide sialique, un lot de CPS 2 dépolymérisée, avec

une taille comparable au lot de CPS désialylée, a été obtenue par ozonolyse selon (291) et a été utilisé pour étudier la reconnaissance des anticorps par dot-blot (**Figure 12**). Les résultats obtenus démontrent à nouveau la dominance du Neu5Ac sur l'épitope dominante reconnue par les sérums anti-sérotype 2, avec peu de différence entre les signaux des CPS native et dépolymérisée. Compte tenu des résultats obtenus avec les CPS désialylées des sérotypes 1 et 14 avec les antisérums contre ces deux réactifs, il apparait peu probable que la perte de reconnaissance de la CPS 2 désialylée puisse être causée par son incapacité à adhérer à la membrane utilisée (**Annexes, ARTICLE X**); cet effet mérite tout de même d'être évalué de manière convenable afin de pouvoir le rejeter hors de tout doute.

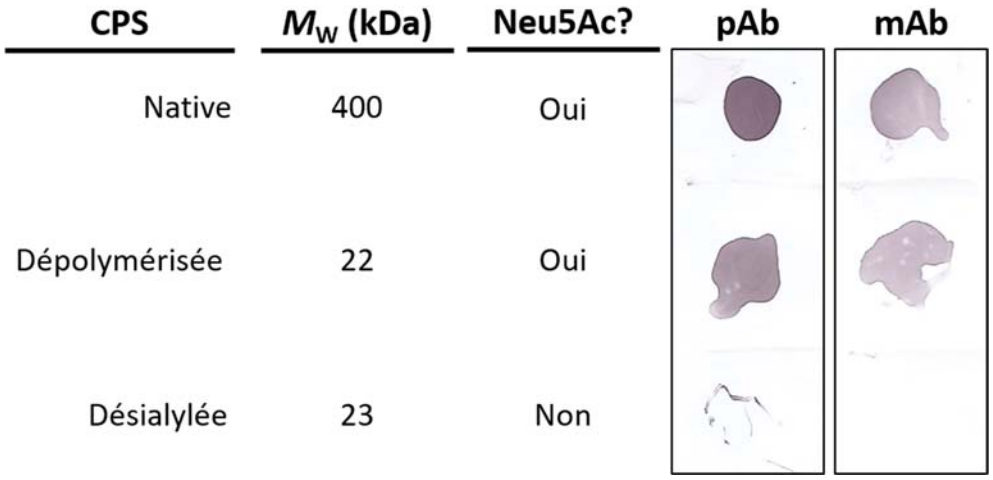


Figure 12. La longueur de la chaîne de la CPS du sérotype 2 n'influence pas sa reconnaissance par des anticorps spécifiques.

Les dot-blots ont été réalisés tel que décrit dans (**Annexes, ARTICLES XIV et XV**). Un sérum de lapin avec une faible dépendance à l'acide sialique (pAb; voir **Figure 11**) et un anticorps monoclonal de souris spécifique pour la CPS 2 [mAb (Z3); provient de (292)] ont été employés. La masse molaire moyenne en masse (M_W) des différentes CPS a été déterminée par analyse de chromatographie d'exclusion stérique couplée à un détecteur de diffusion de lumière multi-angle (SEC-MALS).

Pour en revenir au contrôle conformationnel de l'épitope d'un polysaccharide, celui-ci peut dépendre de la longueur du polysaccharide. Depuis les années 1980, c'était la théorie acceptée pour la CPS du sérotype III de GBS. Celle-ci stipule que l'acide sialique influence et stabilise une conformation hélicoïdale avec les chaînes sialylées pointées de manière

perpendiculaire par rapport à l'axe de l'hélice (173, 293-296). Pour avoir reconnaissance de l'épitope dominante par les anticorps, l'acide sialique et une longueur minimale de deux unités répétitives seraient nécessaires, et une stabilisation de l'épitope surviendrait à partir de 6–7 unités répétitives, résultant en une augmentation de l'affinité qui se maintient jusqu'à 20 unités répétitives, pour ensuite correspondre à l'affinité face à la CPS native. Cependant, une étude publiée en 2017 visant à définir l'épitope minimal protecteur de la CPS du sérotype III de GBS a démontré que l'épitope dominant est en réalité linéaire et constitué d'un total de six sucres répartis sur deux unités répétitives adjacentes, avec notamment la chaîne latérale Neu5Ac-α2,3-Gal- qui est directement impliquée dans la liaison à l'anticorps (297). Basé sur ces nouveaux résultats, les auteurs proposent que la dépendance de l'affinité à la longueur de cette CPS pourrait être expliquée par la multivalence des épitopes plutôt qu'à la nécessité d'adopter une conformation hélicoïdale.

Il n'en reste pas moins que l'épitope majoritaire pour la CPS du sérotype 2 semble dépendre de la présence de l'acide sialique, et que les caractéristiques de cet épitope semblent complexes, sans compter la présence d'un (ou plusieurs) épitope(s) mineur(s). Malheureusement, la CPS dépolymérisée par ozonolyse que nous avons utilisée, avec sa taille de 22 kDa correspondant à une longueur de 17 unités répétitives, ne semble pas être assez courte pour pouvoir évaluer l'effet de la longueur si on se fie au modèle hélicoïdal pour la CPS du sérotype III de GBS. Ceci étant dit, des CPS (ou plutôt des oligosaccharides à cette échelle de grandeur) encore plus courtes devront être obtenues par ozonolyse ou encore par synthèse chimique totale pour produire des longueurs définies (nombre d'unités répétitives).

La question demeure toujours à savoir si l'épitope du sérotype 2 est conformationnel ou linéaire, ainsi que le rôle définitif de l'acide sialique sur celui-ci. Basé sur les résultats obtenus dans le cadre de ces travaux, ainsi que les connaissances concernant les épitopes minimaux des CPS de GBS et de *S. pneumoniae* (297-303), on peut émettre l'hypothèse que l'épitope minimal de la CPS du sérotype 2 de *S. suis* soit formée de quelques monosaccharides contenus dans une à deux unités répétitives et que la chaîne latérale Neu5Ac-α2,6-Gal-β1,4-GlcNAc- soit directement impliquée dans la liaison à l'anticorps. Afin de répondre à cette hypothèse, une approche par chimie synthétique combinée à l'emploi de « glycan arrays » (304) est

requise et pourra être complémentée par d'autres stratégies sophistiquées comme l'utilisation d'un panel d'anticorps monoclonaux (voir plus bas), par modélisation moléculaire (glycochimie computationelle) (305, 306) ou encore par cristallographie aux rayons X (297). Les connaissances concernant les épitopes minimaux protecteurs et opsonisants des anticorps contre les CPS de *S. suis* sont essentielles pour le développement de vaccins efficaces, mais également pour la création de futurs vaccins conjugués synthétiques ou semi-synthétiques.

Un autre rôle important joué par l'acide sialique de *S. suis* est au niveau de la biosynthèse de la CPS, notamment pour le sérotype 2. Des études précédentes ont démontré que la délétion ou l'inactivation (par mutation) des enzymes impliquées dans la synthèse de la chaîne latérale sialylée, de son α2,6-sialyltransférase, des gènes permettant la biosynthèse de l'acide sialique, ou encore de sa flippase (Wzx) et polymérase (Wzy) sont létales : pour survivre, la souche doit alors adopter un phénotype non encapsulé, c'est-à-dire qu'elle doit cesser d'exprimer sa capsule, notamment par l'introduction de mutations ponctuelles (qui peuvent être réversibles *in vivo*) (307, 308) (**Annexes, ARTICLES XIII et XIV**). Additionnellement, une étude plus récente où l'α2,6-sialyltransférase de *S. suis* sérotypes 2 et 14 a été substituée par l'α2,3-sialyltransférase du sérotype III de GBS a également générés des mutants non encapsulés (**Annexes, ARTICLE XIII**). La présence de l'acide sialique et sa liaison α2,6- au Gal semble être critique pour l'expression de la capsule pour les sérotypes sialylés de *S. suis*, notamment le sérotype 2. Cette spécificité proviendrait de la flippase et/ou de la polymérase, enzymes clés dans la biosynthèse des CPS responsables de la reconnaissance de l'unité répétitive formée, de sa translocation vers l'extérieur de la membrane et de son ajout au polysaccharide en cours de synthèse (187).

3.2. Pouvoir activateur des CPS purifiées sur les cellules présentatrices d'antigène

Une étude précédente au laboratoire, pour laquelle je suis co-auteur, visait à étudier l'activation des DCs d'origine murine par les CPS purifiées des sérotypes 2 et 14 de *S. suis*, et plus particulièrement au niveau de la production de cytokines qui sont essentielles au

développement d'une réponse humorale protectrice (309) (**Annexes, ARTICLE XV**). En raison de leur nature glucidique, les CPS bactériennes sont généralement considérées comme faiblement immunogènes comme elles sont incapables de recruter l'aide des cellules T dans l'activation des cellules B, ce qui les classifie comme étant des antigènes TI (207). Alors que plusieurs études *in vitro* ont démontrées les interactions de CPS bactériennes résultant dans la production de cytokines et de chimiokines (310-314), d'autres ont associé cette production avec la présence de contaminants bactériens dans les préparations de CPS « purifiées », notamment des ligands de TLRs (207, 315, 316). La molécule adaptatrice MyD88, qui est impliquée dans la cascade de signalisation intracellulaire suite à l'engagement des TLRs, et le TLR2 sont suspectés d'être impliqués dans les interactions entre les CPS et les CPAs (207, 311, 314, 315). En stimulant les DCs avec les CPS des sérotypes 2 et 14, aucune production de cytokine (pro-inflammatoire ou anti-inflammatoire) n'a été observée, et ceci ne peut être attribué à présence d'acide sialique, un sucre immunomodulateur (**Annexes, ARTICLE XV**). De plus, l'activation de DCs porcines par la CPS 2 n'induit aucune production de cytokine pro-inflammatoire (**Annexes, ARTICLE XVI**). Bien que décevant, ceci indique fort probablement que nos préparations de CPS purifiées sont exemptes de contaminants comme des ligands de TLRs, ce qui est rassurant. Étonnamment, l'activation des DCs par les CPS des sérotypes 2 et 14 de *S. suis* mais également des sérotypes III et V de GBS ont tous induit une importante production des chimiokines CCL2 et CCL3, et ce indépendamment du sérotype (structure) étudiée.

À la suite de la description des structures des CPS pour les sérotypes 3, 7, 8 et 9 plus particulièrement, qui sont structuralement bien différentes de la famille formée par les sérotypes 1, 1/2, 2 et 14, nous avons voulu répéter à nouveau l'étude avec les CPS pour voir si certaines d'entre elles ont le potentiel de moduler différemment l'activation des DCs (**ARTICLE IV**). Aucune des CPS pour les sérotypes 2, 1, 1/2, 3, 7, 8 et 9 n'a induit la production de cytokines pro-inflammatoires, ce qui supporte leur pauvre activité immunostimulatrice. De plus, de fortes productions des chimiokines CCL2 et de CCL3 ont été observées, et ce indépendamment du sérotype (structure) utilisé, avec la CPS du sérotype 19F de *S. pneumoniae* qui fut également utilisée. Tel qu'observé dans l'étude précédente (**Annexes, ARTICLE XV**), la production de CCL2 est indépendante de la voie des TLRs,

alors que la production de CCL3 est partiellement dépendante du TLR2 et de MyD88, quoique non abrogée. Globalement, ces résultats démontrent qu'au moins un autre récepteur cellulaire doit être impliqué dans l'activation des DCs par les CPS, alors que celles-ci répondent d'une manière apparemment indépendante de leurs structures. Ceci nous assure donc de la pertinence d'étudier l'implication potentielle de membres de la grande famille des récepteurs cellulaires de type lectine comprenant, entre autres, les lectines de type C, les galectines et les Siglecs (317-319).

3.3. Immunogénicité des CPS purifiées *in vivo*

Les CPS des sérotypes 2 et 14 de *S. suis* sont connues pour être particulièrement non-immunogènes, et ce même lorsqu'associées à la surface bactérienne dans le cadre d'infections expérimentales (135, 320-323) (**ARTICLE VI**). Encore une fois, cette faible immunogénicité ne peut pas être expliquée par la présence d'acide sialique dans ces deux CPS (134, 322, 324). Toujours selon l'hypothèse que les structures des CPS des sérotypes 3, 7, 8 et 9 peuvent avoir différentes propriétés immunogènes selon leurs structures diversifiées, nous avons choisi d'immuniser les souris avec ces CPS purifiées afin d'évaluer leur immunogénicité *in vivo*. Le modèle d'immunisation choisi fut les souris C57BL/6, une lignée pure de souris permettant de minimiser les variations génétiques d'un individu à l'autre. Ceci nous permet d'étudier les propriétés immunogènes de nos échantillons avec un grand nombre d'animaux sans biais dû à la provenance différente des souris (parents et portée utilisés). Une forte dose de 25 µg de CPS purifiée, le TiterMax Gold® comme adjuvant, la voie d'injection sous-cutanée et le calendrier (immunisation à J0, boost à J21 et sacrifice à J42 pour récolter le sérum) ont été choisis sur la base d'études précédentes sur l'immunogénicité *in vivo* des CPS des sérotypes 2 et 14 de *S. suis* (322) (**ARTICLE VI**).

Compte tenu de leur forte similarité aux CPS des sérotypes 2 et 14 et que celles-ci sont considérées comme étant non immunogènes, il n'a pas été jugé intéressant de tester l'immunogénicité in vivo des CPS des sérotypes 1 et 1/2. Chez les souris immunisées avec les CPS des sérotypes 7, 8 et 9, aucune réponse anticorps anti-CPS n'a pu être détectée (**ARTICLE IV**), ce qui indique que les CPS de ces trois sérotypes sont non-immunogènes

chez la souris. Une limite possible pour ce résultat serait que le modèle utilisé pour l'immunisation ne soit pas optimal pour ces sérotypes; l'influence du modèle d'immunisation sera discutée plus loin dans le cadre de la réponse anti-CPS 2. Il serait pertinent de reproduire cette expérience chez l'hôte naturel (le porc) afin de confirmer ou infirmer l'immunogénicité de ces trois CPS hors de doute.

Au contraire, la CPS 3 adjuvantée avec le TiterMax Gold®, une émulsion eau-dans-huile, induit le développement d'une forte réponse composée d'IgG de type 1 (IgG2b et IgG3 comme sous-classes principales) et d'IgM. Ces niveaux d'anticorps ont été démontrés comme étant opsonisant grâce à un test d'opsonophagocytose (OPA) développé avec du sang entier de souris, lequel sera discuté plus loin, ce qui suggère que ces souris immunisées seraient protégées contre une infection par ce sérotype (**ARTICLE V**). Malgré cela, ceci fait de la CPS 3 la première CPS purifiée de *S. suis* à être décrite comme étant immunogène. L'immunogénicité de la CPS 3, et sa capacité à induire des réponses IgG de type 1, a également été confirmée par l'emploi de la bactérie entière du sérotype 3 de *S. suis* (tuée à la chaleur et vivante) comme antigène (**ARTICLE IV**).

Le modèle actuel pour le développement de l'immunité humorale TI contre les CPS nécessite deux signaux pour assurer la production d'anticorps, la commutation isotypique et le développement d'une mémoire immune (207). Le premier signal est délivré par le cross-linking du récepteur membranaire des cellules B induit par un antigène TI multivalent polymérique (comme une CPS). Le second signal est délivré aux cellules B spécifiques par les cellules de l'immunité innée, comme les cellules NK, grâce à la production de cytokines (comme l'IFN-γ, le « granulocyte-macrophage colony-stimulating factor » (GM-CSF) et l'IL-3) et/ou par la production de « B-cell activating factor » (BAFF)/ « A proliferation-inducing ligand » (APRIL), qui sont induits en réponses aux PAMPs, comme des ligands de TLR. Cependant, dans le cadre des résultats présentés à l'**ARTICLE IV**, nous avons démontrés que nos préparations de CPS sont exemptes de ligands de TLR, de protéines et d'acides nucléiques. Une autre source possible pour le second signal serait l'adjuvant employé : la stimulation de DCs d'origine porcine avec le TiterMax Gold® en absence d'antigène induit la production d'IL-6, alors que la stimulation avec l'Alhydrogel 2%® ou le Quil-A® induit une

production d'IL-6 et/ou d'IL-12, suggérant ainsi que ces trois adjuvants ont la capacité de déclencher le second signal (**Annexes, ARTICLE XVI**). Malgré cela, seule la combinaison de CPS 3 avec le TiterMax Gold® induit une réponse anticorps protectrice. Ceci pourrait être expliqué par l'aspect particulaire d'une émulsion eau-dans-huile. Comparativement aux antigènes solubles, les antigènes particulaires sont phagocytés plus efficacement, induisent des niveaux d'activation immunitaire supérieurs et livrent des antigènes de manière plus concentrée que ce qui peut être obtenue par la pinocytose d'antigènes solubles (325, 326). Il est également à noter que les adjuvants formés de cristaux de sels d'aluminium, comme l'Alhydrogel 2%®, sont en soi particulaires, mais les antigènes qui y sont adsorbés ne se comportent pas comme des antigènes particulaires (325, 326). Malgré tout cela, les propriétés adjuvantes du TiterMax Gold® n'ont pas contribuées à l'immunogénicité des autres CPS étudiées.

Ceci suggère donc que la CPS 3 purifiée possède des propriétés structurales et/ou biochimiques qui lui sont propres. Comme on a remarqué que la CPS 3 précipite en présence de détergent, on suppose que ce polysaccharide possède un caractère amphiphile (présence d'éléments hydrophiles et hydrophobes au sein d'une même molécule) (327). L'élément suspecté d'être hydrophobe serait la di-N-acétylbacillosamine (QuiNAc4NAc), puisque l'acétylation est connue pour accroître l'hydrophobicité d'une molécule [comme c'est le cas pour la chitine versus le chitosane (328)], qui lui est lié à l'acide glucuronique (GlcA; négativement chargé, donc hydrophile). Ensemble, ceux-ci formeraient l'unité répétitive disaccharidique amphiphile de la CPS 3. De ce fait, cet amphiphile peut être coprésenté à l'interface eau-huile et/ou associé avec le copolymère en bloc amphiphile de l'émulsion qu'est le TiterMax Gold®, de manière similaire à sa présentation aux surfaces bactériennes, et bénéficier pleinement de ses propriétés adjuvantes (329). En conclusion, ces résultats forts intéressants soulignent la nécessité d'études plus poussées concernant les propriétés structurales, biochimiques et physicochimiques de la CPS 3, plus particulièrement sur la démonstration de son caractère amphiphile/hydrophobe, de l'influence de celui sur les interactions avec différents adjuvants de type émulsion eau-huile et l'effet global de ceux-ci sur le développement de la réponse humorale.

Pour conclure cette troisième partie sur l'antigénicité et l'immunogénicité des CPSs de *S. suis* :

- Les réactions croisées lors de la sérotypie des souches de *S. suis* entre les sérotypes 1 et 14 et entre les sérotypes 1/2, 1 et 2, ont été expliquées grâce aux structures de leurs CPSs, et sans arriver à définir précisément les épitopes reconnus, les éléments structuraux majeurs et/ou dominants ont pu être identifiés. L'épitope minimal de la CPS du sérotype 2 mérite d'être élucidée pour le développement de vaccins efficaces.

- Par l'étude des interactions des CPS pour les nouveaux sérotypes décrits (3, 7, 8, et 9) avec des cellules présentatrices d'antigène, nous avons confirmé à nouveau que les CPSs de *S. suis* ont un faible pouvoir activateur. Dans la réponse chimiokinique, les CPSs semblent être reconnues « en bloc », indépendamment de leurs différences compositionnelles et/ou structurales : les récepteurs cellulaires impliqués demeurent à être identifiés.

- Par des études d'immunogénicité réalisées *in vivo*, il a été démontré que la CPS du sérotype 3 est la première CPS de *S. suis* à être décrite comme étant immunogénique. Celle-ci se comporte comme un antigène TI classique : cependant, les propriétés uniques de la CPS 3 ainsi que les mécanismes impliqués dans le développement de la réponse humorale TI contre celle-ci demeurent à être définis. Il en demeure que l'utilisation de vaccins, comme des bactérines, pouvant induire une réponse anti-CPS 3 protégeraient contre les infections causées par ce sérotype en médecine porcine.

4. Création d'un vaccin glycoconjugué ciblant la CPS du sérotype 2 de *S. suis* et preuve de concept chez le porc

Compte tenu du fait que le sérotype 2 de *S. suis* est le plus virulent et le plus fréquemment associé à la maladie chez le porc (**ARTICLE I**) et compte tenu de l'absence de vaccins efficaces pour prévenir les infections à *S. suis* commercialement disponibles et de l'efficacité controversée des vaccins de type bactérine présentement employés sur les fermes (133), nous nous sommes intéressés à développer un vaccin permettant de cibler la CPS du sérotype 2. Bien que les CPS bactériennes soient des antigènes TI, des vaccins à base de polysaccharides purifiés existent et sont employés avec succès chez les adultes en médecine humaine (186). L'utilisation de glycoconjugués permet l'emploi de vaccins à base de CPS chez les populations dont le système immunitaire est déficient (enfants en bas âge, aînés et immunocompromis), mais également de CPS non immunogènes en elles-mêmes, par l'induction d'une réponse TD. Ainsi, leur usage est très répandu en médecine humaine. Cependant, les vaccins glycoconjugués demeurent très peu développés en médecine vétérinaire et aucun n'est disponible en médecine porcine.

Pour en revenir à la CPS du sérotype 2, qui rappelons-le est un candidat idéal pour le développement d'un vaccin potentiellement efficace et protecteur, mais qui est aussi particulièrement non immunogène en soi (comparativement à ce qui a déjà été rapporté pour les CPS de *S. pneumoniae* et de GBS) (330-334), notre hypothèse est qu'un vaccin glycoconjugué basé sur la CPS du sérotype 2 produira une réponse anticorps protectrice (**ARTICLE VI**). Comme protéine porteuse dans le glycoconjugué, le TT a été sélectionné pour sa grande immunogénicité et son usage répandu dans les vaccins glycoconjugués (224). De plus, le couplage de la CPS 2 au TT a été réalisé par amination réductrice, une réaction classique pour la création de glycoconjugués (239).

Pour faire la preuve de concept de l'emploi d'un vaccin glycoconjugué pour prévenir les infections par le sérotype 2 de *S. suis*, des porcelets sevrés ont été immunisés puis « challengés » par une infection létale. L'âge des animaux au moment de l'infection était de 8

semaines, ce qui est représentatif de la fenêtre de susceptibilité des porcs qui est de 5 – 10 semaines sur le terrain. En caractérisant la réponse anti-CPS avant boost et avant challenge, nous avons observé une réponse TD induite par notre glycoconjugué qui a également protégé les animaux immunisés. Il est à noter que c'est la première fois qu'une réponse anti-CPS protectrice a été induite chez les porcs.

Ainsi, chez le porc, la production d'IgM et IgG1 anti-CPS aura permis de protéger les porcs vaccinés avec le glycoconjugé. Malgré la controverse entourant l'efficacité de l'IgG1 porcin dans l'opsonophagocytose (Activation du complément? Liaison aux FcγR?), il ne faut pas négliger la contribution de l'IgM, qui à ce jour demeure peu étudié comparativement aux autres isotypes. Leur pentavalence, combinée avec leur faible affinité, leur confère une polyréactivité qui facilite grandement leur capacité à agglutiner les pathogènes, en plus d'être de puissants activateurs du complément (222, 335). Ainsi, les IgM peuvent contribuer de manière considérable à la protection contre une bactérie encapsulée comme *S. suis* (discuté plus loin). La contribution des IgG1 porcins à la protection observée demeure à être évaluée.

Il est crucial de constater ici que le modèle d'infection systémique employé fut particulièrement agressif, et que malgré cela, les niveaux d'anticorps anti-CPS induits par notre vaccin glycoconjugué ont été protecteurs. Ce faisant, alors que la voie naturelle principale d'infection des porcs par *S. suis* se situe au niveau des voies respiratoires, lorsqu'un nombre restreint de bactéries atteindront la circulation sanguine, celles-ci seront éliminées presque immédiatement de la circulation sanguine par les anticorps opsonisants, prévenant ainsi l'établissement d'une infection et l'apparition de signes cliniques.

Dans une perspective de développer un vaccin conférant une protection universelle contre *S. suis*, il serait possible de développer des vaccins multivalents, c'est-à-dire ciblant de multiples sérotypes. Cependant, comme le coût du développement de tels vaccins peut être prohibitif, surtout pour des applications en médecine porcine, cela limiterait au strict minimum le nombre de CPS/glycoconjugués inclus dans la formulation. Une solution à ce problème serait l'utilisation de vaccins semi-synthétiques, où seulement l'épitope minimal pour obtenir des anticorps opsonisants et protecteurs a besoin d'être synthétisé puis conjugué à une protéine

porteuse, permettant de considérablement réduire les coûts de production et d'inclure plus de sérotypes dans la formulation (298-303, 336-339). Ceci étant dit, les épitopes minimaux doivent d'abord être identifiés avant de pouvoir produire ce type de vaccin (discuté plus tôt).

Un des inconvénients des vaccins basés sur la CPS est qu'ils protègent seulement contre les sérotypes inclus dans la formulation. Une autre stratégie est l'emploi de protéines décrites comme antigènes protecteurs universels contre *S. suis* qui pourraient être incluses comme protéine porteuse dans le glycoconjugué, offrant ainsi deux niveaux de protection : un premier contre les sérotypes (CPS) inclus dans la formulation du vaccin, puis un second contre toute souche possédant ladite protéine universelle (sérotypes inclus ou non), comme c'est ce qui est en cours de développement pour de futurs vaccins contre *S. pneumoniae* (186). À titre indicatif, de telles protéines ont été rapportées pour *S. suis*, comme Sao et l'énolase.

Pour conclure cette quatrième partie, nous avons rapporté pour la première fois la conception et la production à l'échelle de laboratoire d'un vaccin glycoconjugué contre le sérotype 2 de *S. suis*. De plus, son utilisation chez le porc a induit une réponse anticorps anti-CPS 2 de nature TD et a conféré une protection significative lors d'un essai de protection *in vivo* avec une souche virulente du sérotype 2 de *S. suis*. Nous avons confirmé l'hypothèse que les vaccins glycoconjugués présentent un fort potentiel pour usage en médecine vétérinaire pour prévenir contre les infections bactériennes invasives.

Ainsi, la voie est maintenant pavée vers l'étude des différents paramètres impliqués dans la création de ce type de vaccin (comme la taille de la CPS, le choix de la chimie de conjugaison, etc.) et leurs effets sur l'efficacité du couplage lors de la conjugaison et sur l'immunogénicité pour établir un vaccin idéal contre le sérotype 2. Également, il serait fort intéressant de développer et d'évaluer des vaccins glycoconjugués mono- et multivalents contre des sérotypes autres que le sérotype 2 de *S. suis*.

5. Immunogénicité d'un vaccin glycoconjugué contre le sérotype 2 de *S. suis* et fonctionnalité des anticorps produits

5.1. Caractéristiques de la réponse humorale

Avec la création du vaccin glycoconjugué, nous avons eu la chance de pouvoir étudier plus précisément son immunogénicité chez la souris, notamment en raison des outils immunologiques disponibles, contrairement au porc. De plus, les études chez la souris permettent de travailler avec un plus grand nombre de d'animaux et de groupes et nécessitent de moins grandes doses vaccinales, ce qui nous permet de pouvoir tester plusieurs variables. Une des premières expériences réalisées chez la souris fut une dose-réponse avec la CPS 2 dépolymérisée (la même CPS qu'utilisée dans la préparation du glycoconjugué) et notre glycoconjugué (ratio 2 CPS : 1 TT). Des doses de 1, 2.5, 5 et 25 µg furent administrées au souris C57BL/6 avec le TiterMax Gold® comme adjuvant, la voie d'injection sous-cutanée et le calendrier (immunisation à J0, boost à J21 et sacrifice à J42 pour récolter le sérum). Alors que les souris immunisées avec la CPS ne semblent produire aucun anticorps anti-CPS, les souris ayant reçu le glycoconjugué produisent une belle cinétique de réponse anti-CPS, sans apparemment atteindre de plateau; c'est pourquoi la dose de 25 µg a été choisie pour les expériences à suivre. Notre hypothèse est que comme l'utilisation d'un vaccin glycoconjugué produit une réponse anti-CPS 2, nous serons alors en mesure de caractériser le profil, la magnitude et la fonctionnalité des anticorps produits.

5.1.1. Magnitude et type de la réponse anti-CPS

L'étude du profil et de la magnitude des isotypes (IgM, IgG, etc.) et des sous-classes (IgG1, IgG2a/IgG2c, IgG2b et IgG3) d'anticorps produits en réponse à un antigène nous permet d'évaluer son immunogénicité. Pour ce qui est d'un vaccin glycoconjugué préparé à partir de CPS, l'étude des isotypes permet d'évaluer si la commutation isotypique des IgM vers les IgG a eu lieu, indicatrice d'une bonne immunogénicité et de la réponse TD désirée. De plus, les différentes sous-classes d'IgG ont des propriétés fonctionnelles différentes; le profil obtenu est donc une indication du type de réponse immunitaire. Les IgGs de type 1 (soit

IgG2b, IgG2c et IgG3 chez la souris) sont associés aux réponses Th1, étant reconnus pour leur capacité à activer le complément et à lier les FcγR, les deux principaux mécanismes permettant l'élimination des bactéries extracellulaires comme *S. suis* par opsonophagocytose. Les IgG de type 2 (soit IgG1 chez la souris) sont associés aux réponses Th2, et sont reconnus pour leur grande affinité et leur capacité à neutraliser toxines et virus. Par contre, les IgG de type 2 sont incapables d'activer le complément ou de déclencher les fonctions effectrices médiées par les FcγR (213).

Tout d'abord, l'immunisation de souris avec 25 µg du glycoconjugué (ratio 2 : 1) a induit de hauts titres d'IgM, d'IgG1, IgG2b, IgG2c et IgG3 (**ARTICLE VI**). Ceci nous indique que ce conjugué possède une excellente immunogénicité. En effet, compte tenu de sa forte immunogénicité, il aurait été possible que le TT puisse agir à titre d'adjuvant seul, ce qui rendrait la conjugaison superflue. Ainsi, notre glycoconjugué adjuvanté avec le TiterMax Gold®, un adjuvant produisant des réponses mixtes Th1/Th2, permet la production de réponses IgGs de type 1 et de type 2 chez la souris.

Il pourrait être intéressant d'étudier davantage les mécanismes sous-tendant la réponse TD suite à l'immunisation avec un vaccin glycoconjugué produit avec une CPS de *S. suis*. Plus particulièrement, il serait fort intéressant de déterminer quel rôle les cellules Tcarb peuvent jouer lors dans cette réponse.

5.1.2. Fonctionnalité des anti-CPS

Dans le contexte du développement de vaccin, un corrélat d'immunité correspond à la mesure d'un paramètre précis qui permet de savoir si un individu est protégé contre une infection, tel qu'un titre d'anticorps spécifique ou un test fonctionnel (340-342). L'utilisation d'un test d'opsonophagocytose (OPA) comme corrélat d'immunité est préféré à un titre d'anticorps spécifique obtenu par « enzyme-linked immunosorbent assay » (ELISA) pour évaluer la protection contre les infections bactériennes invasives.

Grâce au test que nous avons développé à partir de sang entier de souris naïves (**ARTICLE V**), nous avons un modèle réaliste pour évaluer toutes les interactions complexes de l'activité bactéricide du sang. D'ailleurs, ceci est également représentatif de la protection conférée par les anticorps anti-CPS dans le cadre d'une infection systémique par *S. suis*. Ainsi, nous avons pu démontrer que les anticorps produits dans les sérums des souris immunisées avec notre glycoconjugué étaient significativement plus opsonisants que ceux des souris immunisées avec le mélange non conjugué, prouvant la contribution de la conjugaison à l'immunogénicité de notre vaccin glycoconjugué (**ARTICLE VI**). Ce résultat suggère également que ces souris auraient été protégées lors d'un « challenge » par une infection létale, bien que cette protection demeure à être évaluée.

De plus, nous avons également pu utiliser notre test OPA pour évaluer la fonctionnalité d'anticorps dirigés contre la CPS de *S. pneumoniae* (**Annexes, ARTICLES XVII et XVIII**). En effet, un collaborateur travaille à développer des vaccins glycoconjugués de nouvelle génération, nommés « bioconjugués ». Au lieu de se baser sur l'approche chimique classique pour les glycoconjugués (consistant à produire et purifier individuellement les CPS et les protéines porteuses, les modifier [si requis, comme la dépolymérisation des CPS ou la détoxification de toxines], les caractériser individuellement, les conjuguer chimiquement, purifier le glycoconjugué et s'assurer d'éliminer tous les réactifs, puis de finalement caractériser le produit final), cette nouvelle approche repose sur le génie génétique de souches bactériennes pour produire en biofermenteur les CPS couplées enzymatiquement à une composante bactérienne (protéine ou LPS pour former des « outer membrane vesicles », par exemple), qui par la suite nécessite une seule purification suivie d'une seule caractérisation. Dans le cas de structures comme les « outer membrane vesicles », celles-ci sont déjà auto-adjuvantées (contenu en LPS), ce qui peut représenter un avantage économique. Ainsi, grâce au test d'opsonophagocytose à base de sang entier, nous avons pu confirmer que les vaccins glycoconjugués induisent la production d'anticorps anti-CPS opsonisants suite à l'immunisation.

5.2. Influence du choix de l'adjuvant et du modèle animal

Le choix du bon adjuvant est un paramètre souvent critique dans le développement d'un vaccin. Surtout aujourd'hui, avec la préférence accordée aux vaccins sous-unitaires (innocuité et réduction des effets secondaires liées à la vaccination), l'utilisation d'un adjuvant permet de produire une réponse immune plus forte, plus rapide et de meilleure durée. Plus spécifiquement, ils peuvent être utilisés pour diriger le type de réponse immunitaire (Réponse Th1 ou Th2), pour atteindre le seuil de protection plus rapidement (face à une épidémie par exemple), pour réduire la dose administrée et/ou le nombre de doses, ou encore pour administrer le vaccin par une voie alternative (comme les vaccins mucosaux) (241).

Avec notre vaccin glycoconjugué, nous avons démontré que les adjuvants de type émulsion eau-dans-huile (TiterMax Gold® chez la souris et STIMUNE® chez la souris et le porc) produisaient la meilleure immunogénicité, avec des titres d'IgG anti-CPS opsonisants (**ARTICLE VI**). Un autre adjuvant évalué fut le CpG ODN, une version synthétique d'oligonucléotides avec des motifs CpG non méthylés et qui agit comme ligand du TLR9; la dose de 20 µg a été établie selon la littérature (343). Bien que le CpG ODN ait été rapporté comme efficace lors de la vaccination avec des glycoconjugués contre *S. pneumoniae* (344, 345), celui n'a pas réussi à induire de réponse significative avec notre glycoconjugué.

Basés sur les résultats encourageants obtenus précédemment avec les adjuvants d'émulsion eau-dans-huile, nous avons opté pour évaluer d'autres adjuvant de type émulsion eau-huile, soit les Montanide ISA 25 VG® (émulsion huile-dans-eau), ISA 35 VG® (émulsion huile-dans-eau) et ISA 201 VG® (émulsion eau-dans-huile-dans-eau). Malheureusement, aucune réponse anti-CPS n'a pu être détectée avec ces trois adjuvants (**Figure 13**).

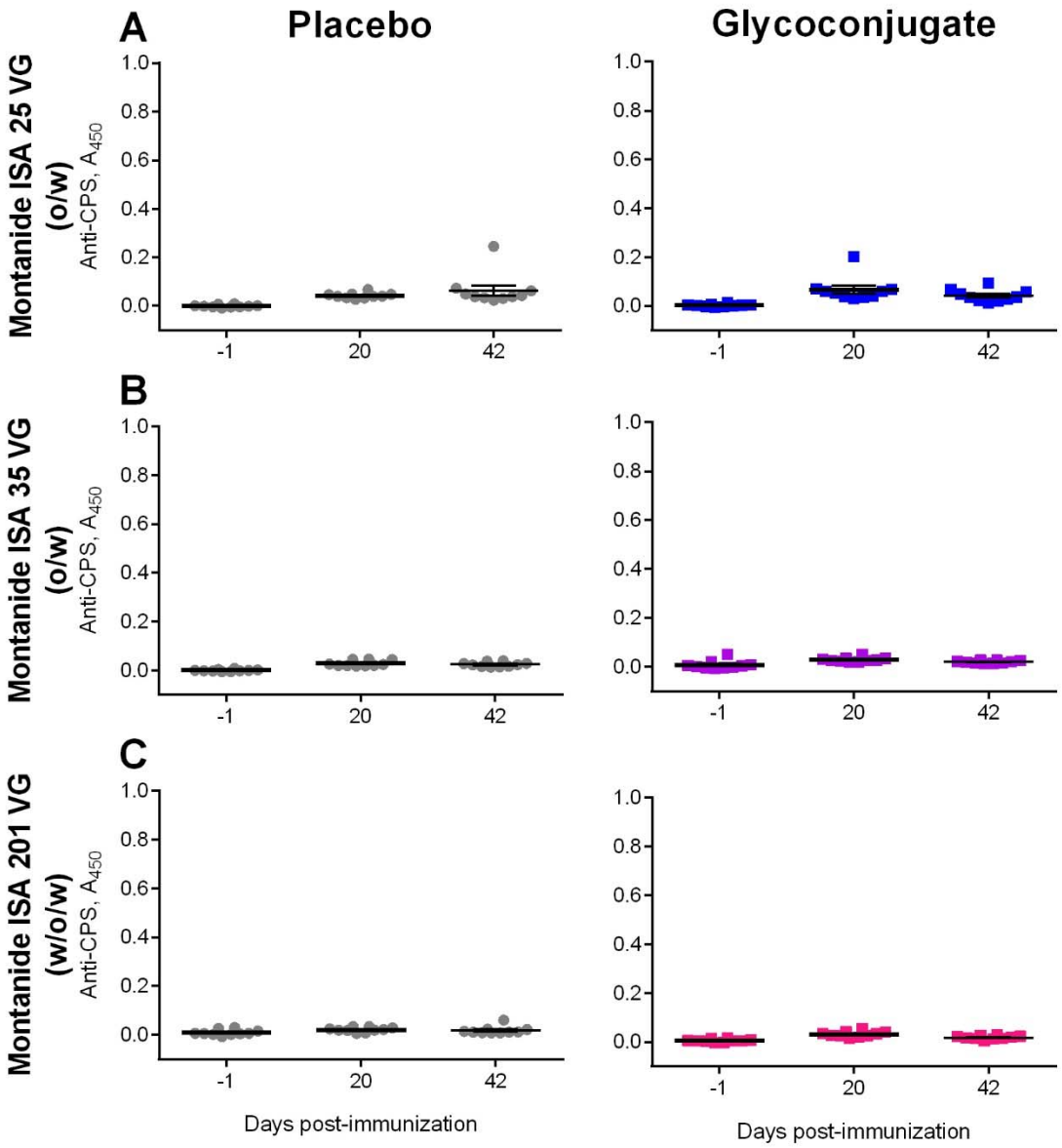


Figure 13. Le glycoconjugué n'induit aucune réponse anticorps anti-CPS lorsqu'adjuvanté avec les Montanide ISA 25 VG® (A), ISA 35 VG® (B) et ISA 201 VG® (C).

Les émulsions ont été préparées selon les recommandations du fabricant, et les immunisations ont été faites telles que précédemment (souris C57BL/6, N = 10, dose de 25 µg de glycoconjugué, injections sous-cutanées à J0 et J21). Les injections pour les groupes placebo ont été préparées de la même façon, mais seulement avec du PBS et l'adjuvant. Les ELISAs anti-CPS ont été réalisés tel que décrit dans (**ARTICLE VI**). Les souris individuelles sont présentées avec la moyenne ± erreur type (SEM). Abréviations : huile-dans-eau (o/w) et eau-dans-huile-dans-eau (w/o/w).

Toujours avec notre glycoconjugué, une expérience additionnelle a été réalisée où nous avons comparé trois différents adjuvants (TiterMax Gold®, émulsion eau-dans-huile; Alhydrogel 2%®, de la famille de l'Alum, utilisé à un ratio de 1 : 1 v/v avec l'antigène tel qu'établi selon la littérature (346, 347); et Quil-A®, de la famille des saponines, utilisé à une dose de 20 µg tel qu'établi selon la littérature (140, 144)) avec trois différentes lignées de souris (C57BL/6, CD-1 et BALB/c). Tel que déjà mentionné, les souris C57BL/6 sont une lignée pure de souris, au même titre que les souris BALB/c; compte tenu de la variabilité génétique minimale de ce type de modèles, un biais génétique est introduit : les souris C57BL/6 sont connues pour avoir un biais envers les réponses Th1 alors que les BALB/c sont connues pour avoir un biais envers les réponses Th2 (348). Au contraire, les souris CD-1 sont un modèle « outbred » permettant d'évaluer la contribution de la diversité génétique au sein d'une population (349). De plus, en ce qui a trait aux sous-classes d'anticorps, les souris BALB/c possèdent l'allèle codant pour l'IgG2a, alors que les souris C57BL/6 quant à elles possèdent l'allèle codant pour l'IgG2c, qui est assumé comme étant fonctionnellement équivalent à l'IgG2a, malgré que cela n'a jamais été formellement étudié (213, 350). Comme les souris CD-1 sont « outbred », donc hétérozygotes pour ces deux allèles, nous avons choisi de mesurer ces deux sous-classes.

Comme on peut le constater à la **Figure 14**, toutes les souris BALB/c (et ce peu importe l'adjuvant utilisé) n'ont pas été en mesure de monter une réponse anticorps à notre vaccin glycoconjugué. Ce fut également le cas pour toutes les souris qui ont reçues notre glycoconjugué avec l'Alhydrogel 2%® comme adjuvant. À titre de contrôle, une vérification des niveaux d'anticorps anti-TT par ELISA [tel que décrit dans (**ARTICLE VI**)] ne montre aucune différence entre les groupes, ce qui indique que les souris et les adjuvants utilisés ont la même capacité pour produire une réponse anti-protéine (résultats non présentés). Comme *S. suis* induit et requiert une immunité Th1 pour son élimination par opsonophagocytose (136, 351) (**ARTICLE VI**), il est fort probable que le biais Th2 des souris BALB/c et des adjuvants de type « Alum » comme l'Alhydrogel 2%® ait nui au développement de la réponse anticorps anti-CPS. L'utilisation du TiterMax Gold® avec des souris CD-1 a produit une réponse positive, mais plus faible que ce qui avait déjà été observé avec le TiterMax Gold® chez les souris C57BL/6 (**ARTICLE VI**). Pour ce qui est du Quil-A®, alors que les souris C57BL/6

n'ont produit aucune réponse anti-CPS, les souris CD-1 quant à elles ont donné une forte réponse comparable à ce qui a été observée avec le TiterMax Gold® chez les souris C57BL/6. Ces conclusions pour les souris CD-1 + TiterMax Gold® et pour les souris CD-1 + Quil-A® sont également maintenues lors de l'étude de la magnitude et du type des réponses anticorps produites, ainsi que par la capacité opsonisante des sérums (**Figure 15**). Il est intéressant de voir que le Quil-A®, un adjuvant avec un biais Th1, permet, en combinaison avec les souris CD-1, d'obtenir une forte réponse anti-CPS opsonisante, et pourrait potentiellement devenir un nouveau modèle d'immunisation pour les études d'immunogénicité avec des vaccins glycoconjugués contre *S. suis*.

Également, il serait intéressant de mentionner qu'une étude *in vitro* réalisée avec des DCs porcines stimulées avec notre glycoconjugué combiné à différents adjuvants a démontré que le Quil-A® influence la réponse cytokinique vers un profil de type 1, contrairement à l'Alhydrogel 2%®, au TiterMax Gold® et au STIMUNE® qui ont influencé la réponse cytokinique vers un profil de type 2 **(Annexes, ARTICLE XVI**).

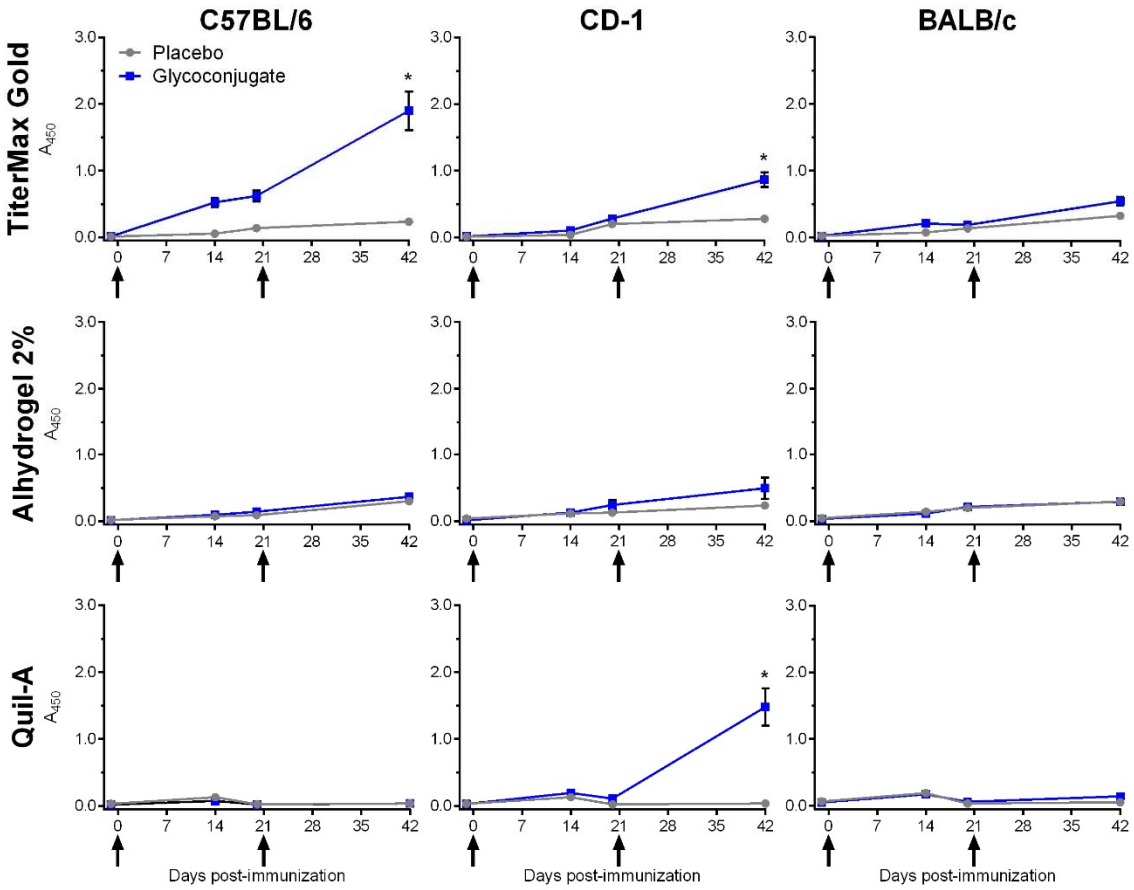


Figure 14. Étude comparative de 3 adjuvants et de 3 lignées de souris sur l'immunogénicité du glycoconjugué contre la CPS du sérotype 2 de *S. suis*.

Les immunisations ont été faites telles que précédemment (N = 10, dose de 25 µg de glycoconjugué, injections sous-cutanées à J0 et J21). La préparation des adjuvants et les doses utilisées sont telles que décrites dans (**ARTICLE IV**). Les injections pour les groupes placebos ont été préparées de la même façon, mais seulement avec du PBS et l'adjuvant. Les ELISAs anti-CPS ont été réalisés tel que décrit dans (**ARTICLE VI**). La valeur moyenne de chaque groupe est montrée ± SEM. * indique une différence significative ($P \leq 0.05$) entre un groupe vacciné et son groupe placebo respectif à l'aide du test de Student. Les flèches indiquent les moments où les souris ont été immunisées.

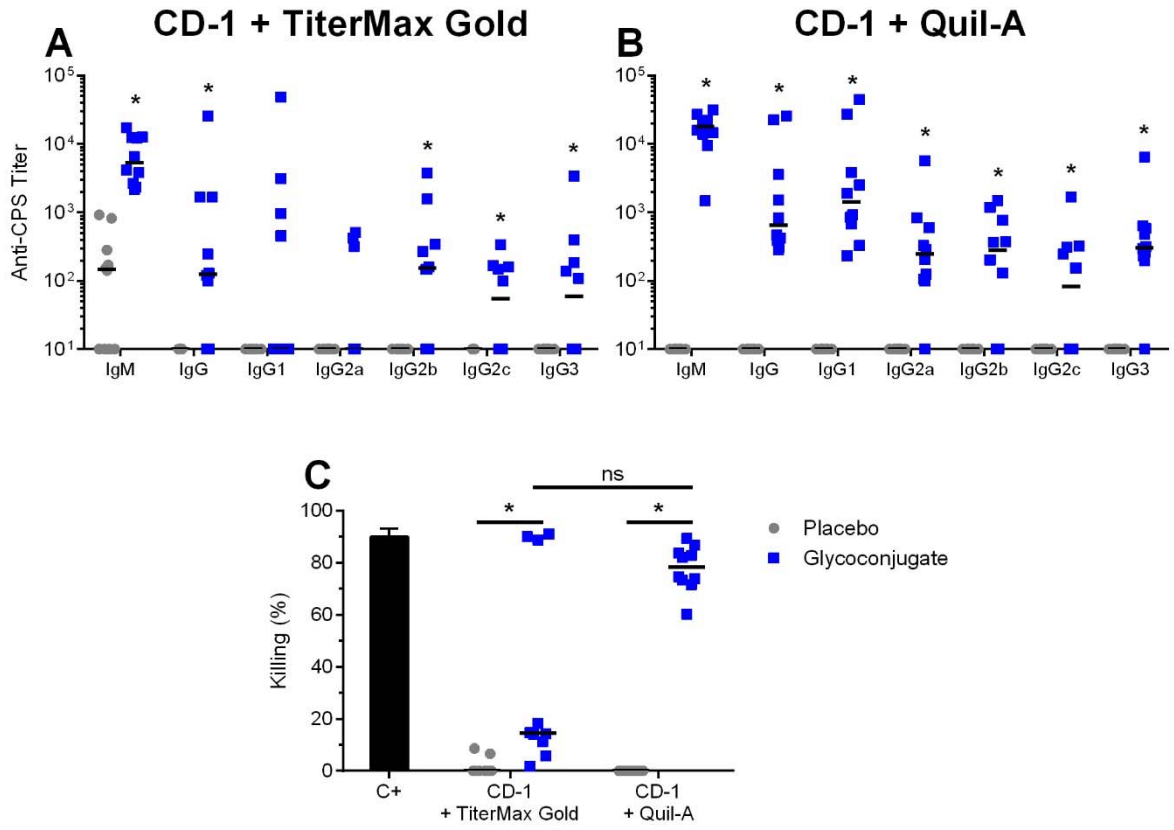


Figure 15. Caractéristiques de la réponse humorale anti-CPS des souris CD-1 ayant reçu le glycoconjugué adjuvanté avec soit le TiterMax Gold® ou Quil-A®.

Analyses réalisées sur les sérums obtenus au J42 (N = 10) de l'immunisation présentée à la **Figure 14**. Magnitude et type de la réponse anticorps des souris CD-1 immunisées avec le glycoconjugué contre la CPS du sérotype 2 de *S. suis* et adjuvanté soit avec le TiterMax Gold® (A) ou avec le Quil-A® (B). La titration des isotypes et des sous-classes d'anticorps anti-CPS 2 a été réalisée telle que décrite dans (**ARTICLES IV et VI**). (C) Fonctionnalité des anticorps anti-CPS induit par immunisation évaluée par le test OPA contre la souche S735 du sérotype 2 de *S. suis*. Le test OPA a été réalisé tel que décrit dans (**ARTICLES V et VI**). Le contrôle positif (C+) utilisé est un antisérum de lapin spécifique contre le sérotype 2 de *S. suis*. Les souris individuelles sont présentées avec la médiane. ns indique l'absence de différence significative (P > 0.05); * indique une différence significative ($P \leq 0.05$), déterminées à l'aide du test de Mann-Whitney, entre un groupe vacciné et son groupe placebo respectif, à moins d'indication contraire.

5.3. Spécificité et potentiel protecteur d'anticorps monoclonaux anti-CPS

En 1997, Charland et al. ont décrit pour la première fois un anticorps monoclonal (mAb) obtenu contre la CPS du sérotype 2 de *S. suis* (292). Des souris BALB/c furent immunisées à répétition avec une souche formolée de sérotype 2 (S735) avec du Quil-A® comme adjuvant. Suite à l'obtention et à la sélection des hybridomes par ELISA anti-CPS 2, seul le mAb Z3 a pu être identifié. Celui-ci fut initialement rapporté comme étant un IgG2b (292), mais on s'est rendu compte plus tard que son isotype était IgM en réalité (**ARTICLE VII**). De plus, le mAb Z3 est spécifique pour les CPS sialylées des sérotypes 2, 1 et 1/2; par oxydation au periodate (affecte principalement le Neu5Ac) et à la sialidase (enzyme hydrolysant les Neu5Ac), il a été démontré que cette reconnaissance dépend entièrement de la présence et de l'intégrité de l'acide sialique pour les sérotypes reconnus. De plus, le potentiel opsonisant et la protection conférée par ce mAb dans un modèle d'immunisation passive de souris ont été démontrés.

Basé sur les fortes réponses IgM et IgG anti-CPS telles qu'obtenues avec notre glycoconjugué (**ARTICLE VI**), celui-ci était donc l'antigène idéal à utiliser pour obtenir et caractériser de nouveaux mAb contre la CPS du sérotype 2. Nous nous en sommes donc servis pour immuniser des souris BALB/c en utilisant le TiterMax Gold® comme adjuvant pour produire les hybridomes, et identifier ceux produisant des mAb spécifiques à la CPS sialylée du sérotype 2. De cette expérience, deux nouveaux IgM (mAb 9E7 et 13C8) et un IgG1 (mAb 16H11) ont été identifiés (**ARTICLE VII**). De plus, la reconnaissance de ces anticorps aux différentes CPS testées (selon leur spécificité individuelle) dépend absolument de la présence de l'acide sialique, comme ce qui a été rapporté précédemment pour le mAb Z3 (292), mais également pour l'épitope majoritaire de la CPS 2 reconnue par les antisérums de lapin (**Annexes, ARTICLE X**).

En rétrospective, basé sur les résultats présentés à la **Figure 14** qui montrent que les souris BALB/c répondent peu ou pas du tout à notre vaccin glycoconjugué, nous pouvons conclure que cette lignée de souris n'est pas la lignée idéale pour la production d'anticorps monoclonaux anti-CPS 2. Ceci étant dit, la lignée cellulaire utilisée (le myélome SP2/0) est la seule disponible au laboratoire comme partenaire de fusion, et ce faisant, cette lignée provient

de cellules spléniques immortalisées de souris BALB/c (352). Malgré cela, il serait possible d'immuniser des souris C57BL/6 (excellent modèle pour notre glycoconjugué) et d'utiliser un partenaire de fusion provenant de BALB/c (353) : ceci aurait fort probablement contribué à l'obtention d'un plus grand nombre de clones et d'IgGs.

Malgré tout, la stratégie employée nous a quand même permis d'obtenir un IgG1 murin (mAb 16H11) spécifique seulement pour la CPS du sérotype 2 de *S. suis*. Cette sous-classe d'anticorps est surtout privilégiée lors des réponses de type Th2. Ce biais aurait pu provenir de la stratégie d'immunisation, de la génétique des souris immunisées (BALB/c), des propriétés immunologiques du TT utilisé pour notre glycoconjugué et/ou de l'adjuvant utilisé. Comme les réponses anticorps TD sont connues pour induire le processus de maturation d'affinité lors de la réaction germinale, ceci permettrait d'expliquer sa spécificité unique pour la CPS du sérotype 2. Comme le mAb 16H11 ne peut reconnaître la CPS du sérotype 1/2, cela fait de lui le premier anticorps à être rapporté comme étant spécifique seulement pour la CPS du sérotype 2. Actuellement, comme la sérotypie par PCR ne peut distinguer entre un sérotype 2 et un sérotype 1/2, il faut absolument utiliser à la fois les antisérums de lapin contre les sérotypes 1 et 2 pour bien identifier la souche. Ceci fait donc du mAb 16H11 un outil prometteur pour la sérotypie et l'identification de souches du sérotype 2 en une seule réaction. Néanmoins, en raison de sa nature de sous-classe d'IgG de type 2, ce mAb n'a aucun potentiel protecteur, puisqu'il est incapable d'induire l'élimination de *S. suis* par opsonophagocytose *in vitro* ou de protéger *in vivo*. Une perspective intéressante pour obtenir de nouveaux mAb de la sous-classe des IgGs de type 1 (opsonisants et potentiellement protecteurs) serait par l'induction *in vitro* de la commutation isotypique des hybridomes en culture (354, 355).

Contrairement à l'IgG1, les deux IgMs murins que nous avons décrits (mAb 9E7 et 13C8) ont été démontrés comme étant opsonisants *in vitro*. Cependant, dans notre modèle d'immunisation passive, le mAb 9E7 protège contre l'infection avec une dose létale du sérotype 2 de *S. suis* (de manière similaire au mAb Z3), alors que le mAb 13C8 ne confère aucune protection significative. L'explication la plus probable concernant les différences de protection entre les mAb 9E7/Z3 et le mAb 13C8 se situerait au niveau de leur spécificité : les mAb 9E7 (reconnait les sérotypes 1, 1/2, 2 et 14) et Z3 (reconnait les sérotypes 1, 1/2 et 2)

sont considérés comme étant plus « poly-réactifs » que le mAb 13C8 (reconnait les sérotypes 2 et 14). Cette « poly-réactivité » des mAb 9E7/Z3 s'illustre notamment au niveau de leurs indices d'opsonisation (tels que déterminés lors des expériences de dose-réponse lors du test OPA), de leurs capacités à agglutiner *S. suis* sérotype 2 et de leurs capacités à réduire la bactériémie lors de l'infection avec la dose létale suite à l'immunisation passive. Dans les faits, l'agglutination médiée par les anticorps (notamment IgM, mais aussi IgG et IgA) favorise grandement l'élimination des pathogènes et prévient la colonisation et/ou l'infection (335, 356). Des études avec *S. pneumoniae* ont permis de démontrer comment son agglutination par des anticorps et l'activation résultante du complément mène à son élimination par phagocytose (357-360). De plus, le mAb 13C8, bien que non significativement protecteur *in vivo* et incapable d'agglutiner *S. suis* sérotype 2, a quand même été démontré comme étant opsonisant lors du test OPA et permettant la phagocytose de *S. suis* sérotype 2 tel que démontré par microscopie confocale. Ceci illustre bien la capacité opsonisante des IgM via l'activation du complément et l'engagement des récepteurs du complément pour induire la phagocytose (361). Ainsi, grâce à l'obtention de ces mAb IgM anti-CPS, nous avons pu confirmer que cet isotype d'anticorps est opsonisant et facilite l'élimination bactérienne par opsonophagocytose, et donc, par extension, que les IgMs peuvent jouer un rôle protecteur contre *S. suis*. De plus, cette étude illustre à nouveau le grand potentiel des CPS comme antigènes vaccinaux pour la prévention des infections causées par des bactéries encapsulées extracellulaires.

Pour de futures études sur les fonctions des anticorps anti-CPS contre *S. suis*, il pourrait être fort intéressant de produire des anticorps monoclonaux d'isotype et de sous-classes d'IgG différents, mais idéalement possédant tous la même affinité pour le même épitope. Ceci permettra d'évaluer la contribution individuelle de chaque isotype/sous-classe à la protection et à l'élimination de *S. suis*. De plus, alors que les résultats obtenus suggèrent fortement une contribution importante de la voie du complément dans l'élimination de *S. suis* par opsonophagocytose, celle-ci n'a pas été évaluée et mériterait d'être étudiée dans le futur.

Pour conclure cette cinquième partie sur l'immunogénicité du vaccin glycoconjugué contre le sérotype 2 de *S. suis* et la fonctionnalité des anticorps produits :

- Nous avons observé que l'utilisation du glycoconjugué chez la souris permettait d'induire une réponse anticorps de nature TD, avec la production d'IgM, d'IgGs de type 1 (IgG2a/2c, IgG2b, IgG3) et d'IgG de type 2 (IgG1), et que ces anticorps sont fonctionnels (et potentiellement protecteurs) par leur capacité à induire l'élimination de *S. suis* par opsonophagocytose.

- Nous avons également observé que le choix de l'adjuvant et du modèle animal utilisé influence grandement l'immunogénicité observée du glycoconjugué. L'utilisation d'adjuvants de type émulsion eau-dans-huile ou de type saponine, lorsque combiné à une lignée de souris appropriée, permet d'obtenir d'excellentes réponses vaccinales.

- Nous avons rapporté l'obtention de 3 nouveaux anticorps monoclonaux murins dirigés contre la CPS du sérotype 2 de *S. suis* : un IgG1 reconnaissant exclusivement la CPS sialylée du sérotype 2 et deux IgM aux spécificités de sérotypes et aux propriétés effectrices différentes qui ont permis d'étudier les mécanismes impliqués dans l'élimination de *S. suis* par opsonophagocytose.

Ainsi, l'utilisation d'un vaccin glycoconjugué rend la CPS du sérotype 2 de S. suis immunogénique et nous a permis de caractériser le profil, la magnitude et la fonctionnalité des anticorps produits chez des souris immunisées. Le glycoconjugué a également permis l'obtention de nouveaux mAb, et l'étude de ceux-ci nous a permis de mieux caractériser les mécanismes impliqués dans l'élimination de *S. suis* par opsonophagocytose.

La **Figure 16** présentée à la page suivante propose un modèle des mécanismes du développement de la réponse humorale dirigée contre les CPS de *S. suis* et de la protection médiée par ces anticorps anti-CPS selon les résultats obtenus dans le cadre de cette thèse et ce qui a été rapporté dans la littérature.

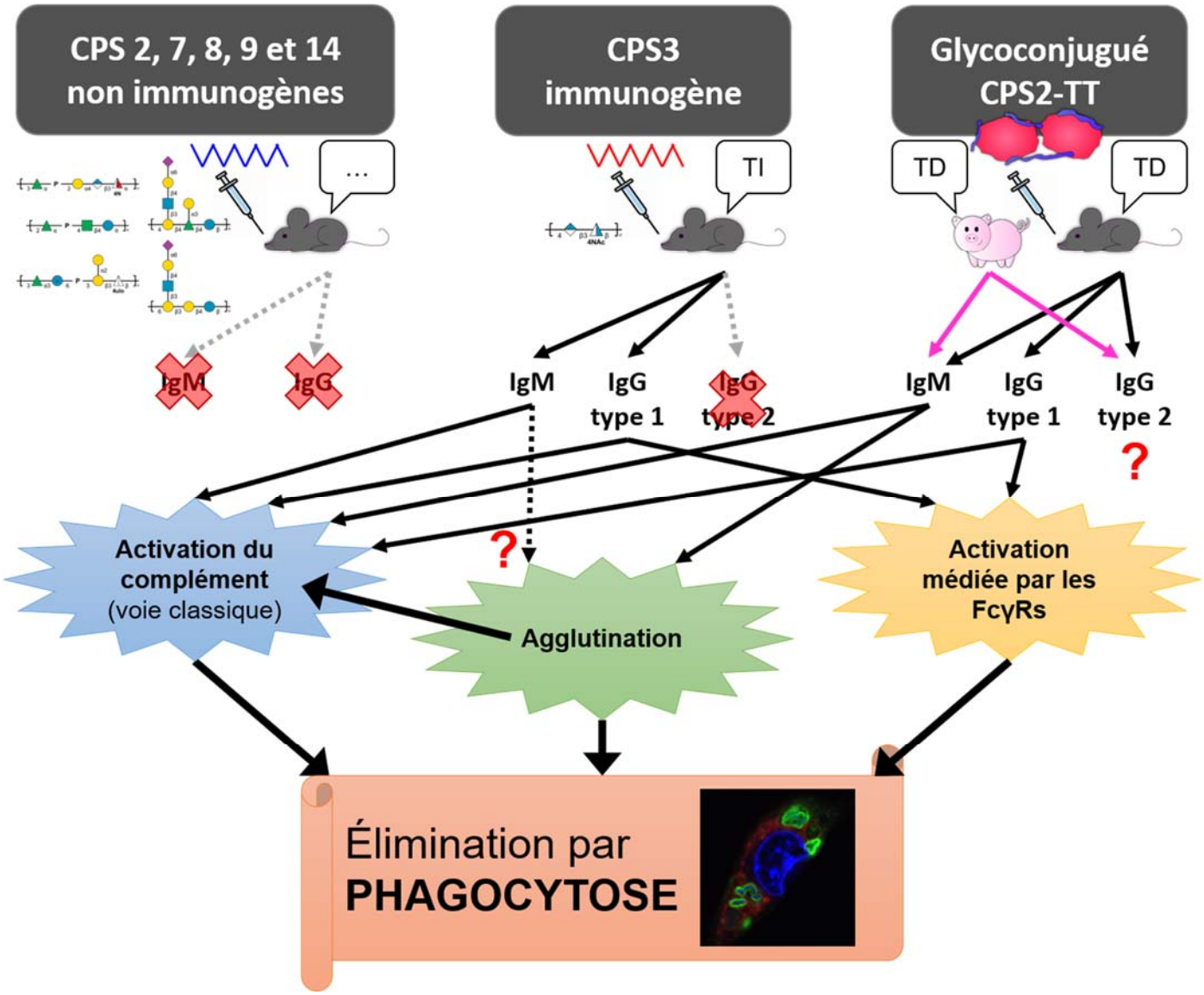


Figure 16. Modèle proposé des mécanismes du développement de la réponse humorale dirigée contre les CPS de *S. suis* et de la protection médiée par ces anticorps anti-CPS.

(1) Contrairement à la plupart des CPS purifiées testées qui sont non immunogène, c'est-à-dire qu'il n'y a aucun ou très peu d'anticorps spécifiques induit suite à la vaccination, il a été observé que les souris vaccinées avec la

CPS purifiée du sérotype 3 de *S. suis* produisent une réponse TI classique opsonisante. **(2)** Pour réussir à obtenir une réponse anticorps contre une CPS non immunogène comme celle du sérotype 2 de *S. suis*, la création et l'utilisation d'un vaccin glycoconjugué permet l'induction d'une réponse TD opsonisante chez la souris et protectrice chez le porc. **(3)** En absence d'anticorps spécifiques, cette bactérie encapsulée est résistante à la phagocytose et au complément. L'opsonisation par les IgG de type 1 provoque la phagocytose médiée par les FcγR qui est hautement efficace dans l'élimination de *S. suis*. De plus, ceux-ci déclenchent également l'activation du complément, mécanisme synergique aux FcγR pour l'élimination de *S. suis* par phagocytose. **(4)** Les IgM dirigés contre la CPS peuvent être également protéger en agglutinant les bactéries; celle-ci facilite considérablement le travail des phagocytes en plus de pouvoir activer la voie du complément. D'autant plus, les IgM sont de puissants activateurs du complément une fois à la surface du pathogène. **(5)** Ainsi, suite à l'agglutination de *S. suis* et/ou l'opsonisation par les anticorps et le complément, les phagocytes verront leurs activités phagocytaires, bactéricides et protéolytiques accrues pour faciliter l'élimination du pathogène. **(6)** Pour conclure, bien que difficiles à obtenir, les anticorps spécifiques à la CPS sont efficaces pour prévenir et combattre les infections à *S. suis*. Ceci fait que les CPS sont des antigènes prometteurs pour le développement de vaccins efficaces contre ce pathogène.

Abréviations : CPS, capsule polysaccharidique; FcγR, récepteur Fcγ; IgG, immunoglobuline G; IgM, immunoglobuline M; TD, réponse T-dépendante; TI, réponse T-indépendante; TT, toxoïde tétanique.

V. CONCLUSIONS GÉNÉRALES ET PERSPECTIVES

Dans le cadre de cette thèse, nous nous sommes d'abord intéressés à la diversité des CPS de *S. suis*. Dans un premier temps, un portrait global et à jour des sérotypes de *S. suis* les plus importants impliqués dans les infections chez le porc et chez l'homme a été présenté, compte tenu du fait que cette analyse n'avait pas été faite depuis fort longtemps. Dans un second temps, nous avons réussi à purifier les CPS et à élucider les structures des unités répétitives pour huit sérotypes de *S. suis*, soit les sérotypes 1, 1/2, 3, 7, 8, 9, 14 et 18, qui demeuraient jusqu'alors inconnues. En effet, l'élucidation des structures de CPS constitue la première étape de toute démarche visant la création et l'étude de vaccins basés sur les CPS, et justifie de poursuivre l'élucidation des structures pour tous les 35 sérotypes de *S. suis*.

Avec les CPS dont les structures sont connues, nous nous sommes ensuite intéressés aux caractéristiques de la réponse humorale face aux CPS de *S. suis*. Dans un troisième temps, l'étude de l'antigénicité des CPS, et plus particulièrement du sérotype 2, a permis d'identifier les principaux éléments structuraux reconnus par les anticorps. Cependant, l'identification de l'épitope minimal mènera au développement de futurs vaccins synthétiques. De plus, une CPS immunogène, soit celle du sérotype 3, a été rapportée chez *S. suis* pour la première fois; en définissant mieux ses propriétés immunogènes, il sera possible de pouvoir l'utiliser tel quel comme antigène vaccinal. Dans un quatrième temps, la création d'un vaccin glycoconjugué à partir de la CPS du sérotype 2 et son utilisation chez des porcs a démontré pour la première fois l'efficacité de la CPS pour protéger contre les infections à *S. suis* sérotype 2, et plus globablement le potentiel des vaccins glycoconjugués en général pour usage vétérinaire. Dans un cinquième temps, l'étude du vaccin glycoconjugué ciblant le sérotype 2 de *S. suis* chez la souris a permis de souligner l'importance du choix de l'adjuvant et du modèle animal utilisé sur les caractéristiques de la réponse anticorps produite. Par l'obtention de nouveaux mAb, ces nouveaux outils sérologiques nous ont également permis de mieux caractériser les mécanismes impliqués dans l'élimination de *S. suis* par opsonophagocytose. Il serait intéressant de poursuivre ces études afin de déterminer la contribution des différents types d'anticorps ainsi que de la voie du complément dans l'élimination de *S. suis* par les anticorps anti-CPS.

Pour conclure, bien que difficiles à obtenir, les anticorps spécifiques à la CPS sont efficaces pour prévenir et combattre les infections à *S. suis* en permettant son élimination par

opsonophagocytose, tel que démontré avec la CPS immunogène du sérotype 3 et le vaccin glycoconjugué produit à partir de la CPS du sérotype 2. Ceci fait que les CPS sont des antigènes prometteurs pour le développement de vaccins efficaces contre ce pathogène.

VI. BIBLIOGRAPHIE

1. Gottschalk M, Xu J, Calzas C, Segura M. *Streptococcus suis*: a new emerging or an old neglected zoonotic pathogen? Future Microbiol. 2010;5(3):371-91.

2. Feng Y, Zhang H, Wu Z, Wang S, Cao M, Hu D, et al. *Streptococcus suis* infection: an emerging/reemerging challenge of bacterial infectious diseases? Virulence. 2014;5(4):477-97.

3. Gottschalk M. Streptococcosis. In: Zimmerman JJ, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of swine. 10th ed. Ames, USA: Wiley-Blackwell Publishing; 2012. p. 841-55.

4. Baele M, Chiers K, Devriese LA, Smith HE, Wisselink HJ, Vaneechoutte M, et al. The gram-positive tonsillar and nasal flora of piglets before and after weaning. J Appl Microbiol. 2001;91(6):997-1003.

5. Reams RY, Harrington DD, Glickman LT, Thacker HL, Bowersock TL. Multiple serotypes and strains of *Streptococcus suis* in naturally infected swine herds. J Vet Diag Invest. 1996;8(1):119-21.

6. Thanawongnuwech R, Brown GB, Halbur PG, Roth JA, Royer RL, Thacker BJ. Pathogenesis of porcine reproductive and respiratory syndrome virus-induced increase in susceptibility to *Streptococcus suis* infection. Vet Pathol. 2000;37(2):143-52.

7. Vötsch D, Willenborg M, Weldearegay YB, Valentin-Weigand P. *Streptococcus suis* – The “two faces” of a pathobiont in the porcine respiratory tract. Frontiers in microbiology. 2018;9:480.

8. Gottschalk M, Segura M, Xu J. *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. Anim Health Res Rev. 2007;8(1):29-45.

9. Staats JJ, Feder I, Okwumabua O, Chengappa MM. *Streptococcus suis*: past and present. Vet Res Commun. 1997;21(6):381-407.

10. Su Y, Yao W, Perez-Gutierrez ON, Smidt H, Zhu WY. Changes in abundance of *Lactobacillus* spp. and *Streptococcus suis* in the stomach, jejunum and ileum of piglets after weaning. FEMS microbiology ecology. 2008;66(3):546-55.

11. Bahloul H, Mofredj A, Mrabet A, Gineyt G, Rousselier P. *Streptococcus suis* meningitis after oral contamination? Med Mal Infect. 2008;38(5):281-2.

12. Goyette-Desjardins G, Auger J-P, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect. 2014;3(6):e45.

13. Lun Z, Wang Q, Chen X, Li A, Zhu X. *Streptococcus suis*: an emerging zoonotic pathogen. Lancet Infect Dis. 2007;7(3):201-9.

14. Huh HJ, Park KJ, Jang JH, Lee M, Lee JH, Ahn YH, et al. *Streptococcus suis* meningitis with bilateral sensorineural hearing loss. Korean J Lab Med. 2011;31(3):205-11.

15. Gottschalk M, Higgins R, Jacques M, Mittal KR, Henrichsen J. Description of 14 new capsular types of *Streptococcus suis*. J Clin Microbiol. 1989;27(12):2633-6.

16. Gottschalk M, Higgins R, Boudreau M. Use of polyvalent coagglutination reagents for serotyping of *Streptococcus suis*. J Clin Microbiol. 1993;31(8):2192-4.

17. Perch B, Kjems E, Slot P, Pedersen KB. Biochemical and serological properties of R, S, and RS streptococci. Acta Pathol Microbiol Scand B. 1981;89(3):167-71.

18. Perch B, Pedersen KB, Henrichsen J. Serology of capsulated streptococci pathogenic for pigs: six new serotypes of *Streptococcus suis*. J Clin Microbiol. 1983;17(6):993-6.

19. Gottschalk M, Higgins R, Jacques M, Beaudoin M, Henrichsen J. Characterization of six new capsular types (23 through 28) of *Streptococcus suis*. J Clin Microbiol. 1991;29(11):2590-4.

20. Higgins R, Gottschalk M, Boudreau M, Lebrun A, Henrichsen J. Description of six new capsular types (29-34) of *Streptococcus suis*. J Vet Diagn Invest. 1995;7(3):405-6.

21. Zheng H, Ji S, Liu Z, Lan R, Huang Y, Bai X, et al. Eight Novel Capsular Polysaccharide Synthesis Gene Loci Identified in Nontypeable Streptococcus suis Isolates. Appl Environ Microbiol. 2015;81(12):4111-9.

22. Gottschalk M, Lacouture S, Bonifait L, Roy D, Fittipaldi N, Grenier D. Characterization of *Streptococcus suis* isolates recovered between 2008 and 2011 from diseased pigs in Quebec, Canada. Vet Microbiol. 2013;162(2-4):819-25.

23. Van Calsteren MR, Goyette-Desjardins G, Gagnon F, Okura M, Takamatsu D, Roy R, et al. Explaining the serological characteristics of *Streptococcus suis* serotypes 1 and 1/2 from their capsular polysaccharide structure and biosynthesis. J Biol Chem. 2016.

24. Zheng H, Ji S, Liu Z, Lan R, Huang Y, Bai X, et al. Identification of 8 novel capsular polysaccharide synthesis gene loci in non-typable *Streptococcus suis*. Appl Environ Microbiol. 2015.

25. Chatellier S, Harel J, Zhang Y, Gottschalk M, Higgins R, Devriese LA, et al. Phylogenetic diversity of Streptococcus suis strains of various serotypes as revealed by 16S rRNA gene sequence comparison. Int J Syst Bacteriol. 1998;48 Pt 2:581-9.

26. Tien LHT, Nishibori T, Nishitani Y, Nomoto R, Osawa R. Reappraisal of the taxonomy of Streptococcus suis serotypes 20, 22, 26, and 33 based on DNA–DNA homology and sodA and recN phylogenies. Vet Microbiol. 2013;162(2–4):842-9.

27. Segura M, Zheng H, Greeff A, Gao GF, Grenier D, Jiang Y, et al. Latest developments on *Streptococcus suis*: an emerging zoonotic pathogen: part 1. Future Microbiol. 2014;9:441-4.

28. Okura M, Osaki M, Nomoto R, Arai S, Osawa R, Sekizaki T, et al. Current taxonomical stuation of *Streptococcus suis*. Pathogens. 2016;5(3).

29. Nomoto R, Maruyama F, Ishida S, Tohya M, Sekizaki T, Osawa R. Reappraisal of the taxonomy of Streptococcus suis serotypes 20, 22 and 26: Streptococcus parasuis sp. nov. Int J Syst Evol Microbiol. 2015;65(Pt 2):438-43.

30. Tohya M, Arai S, Tomida J, Watanabe T, Kawamura Y, Katsumi M, et al. Defining the taxonomic status of Streptococcus suis serotype 33: the proposal for Streptococcus ruminantium sp. nov. Int J Syst Evol Microbiol. 2017;67(9):3660-5.

31. Hill JE, Gottschalk M, Brousseau R, Harel J, Hemmingsen SM, Goh SH. Biochemical analysis, cpn60 and 16S rDNA sequence data indicate that *Streptococcus suis* serotypes 32 and 34, isolated from pigs, are *Streptococcus orisratti*. Vet Microbiol. 2005;107(1–2):63-9.

32. Okura M, Takamatsu D, Maruyama F, Nozawa T, Nakagawa I, Osaki M, et al. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: Potential mechanisms for the generation of capsular variation. Appl Environ Microbiol. 2013.

33. Okura M, Lachance C, Osaki M, Sekizaki T, Maruyama F, Nozawa T, et al. Development of a two-step multiplex PCR assay for typing of capsular polysaccharide synthesis gene clusters of *Streptococcus suis*. J Clin Microbiol. 2014;52(5):1714-9.

34. Pan ZH, Ma JL, Dong WY, Song WC, Wang KC, Lu CP, et al. Novel Variant Serotype of Streptococcus suis Isolated from Piglets with Meningitis. Appl Environ Microbiol. 2015;81(3):976-85.

35. Qiu X, Bai X, Lan R, Zheng H, Xu J. Novel capsular polysaccharide Loci and new diagnostic tools for high-throughput capsular gene typing in Streptococcus suis. Appl Environ Microbiol. 2016.

36. Zheng H, Qiu XT, Roy D, Segura M, Du PC, Xu JG, et al. Genotyping and investigating capsular polysaccharide synthesis gene loci of non-serotypeable Streptococcus suis isolated from diseased pigs in Canada. Vet Res. 2017;48.

37. Brehony C, Jolley KA, Maiden MC. Multilocus sequence typing for global surveillance of meningococcal disease. FEMS Microbiol Rev. 2007;31(1):15-26.

38. King SJ, Leigh JA, Heath PJ, Luque I, Tarradas C, Dowson CG, et al. Development of a multilocus sequence typing scheme for the pig pathogen *Streptococcus suis*:

identification of virulent clones and potential capsular serotype exchange. J Clin Microbiol. 2002;40(10):3671-80.

39. Fittipaldi N, Xu J, Lacouture S, Tharavichitkul P, Osaki M, Sekizaki T, et al. Lineage and virulence of *Streptococcus suis* serotype 2 isolates from North America. Emerg Infect Dis. 2011;17(12):2239-44.

40. Lun ZR, Wang QP, Chen XG, Li AX, Zhu XQ. *Streptococcus suis*: an emerging zoonotic pathogen. Lancet Infect Dis. 2007;7(3):201-9.

41. Wertheim HF, Nghia HD, Taylor W, Schultsz C. *Streptococcus suis*: an emerging human pathogen. Clin Infect Dis. 2009;48(5):617-25.

42. Fittipaldi N, Segura M, Grenier D, Gottschalk M. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent *Streptococcus suis*. Future Microbiol. 2012;7(2):259-79.

43. Wilson SM, Norton P, Haverson K, Leigh J, Bailey M. Interactions between *Streptococcus suis* serotype 2 and cells of the myeloid lineage in the palatine tonsil of the pig. Vet Immunol Immunopathol. 2007;117(1-2):116-23.

44. Madsen LW, Bak H, Nielsen B, Jensen HE, Aalbaek B, Riising HJ. Bacterial colonization and invasion in pigs experimentally exposed to *Streptococcus suis* serotype 2 in aerosol. J Vet Med. 2002;49(5):211-5.

45. Segura M, Calzas C, Grenier D, Gottschalk M. Initial steps of the pathogenesis of the infection caused by *Streptococcus suis*: fighting against nonspecific defenses. FEBS Lett. 2016;590(21):3772-99.

46. Chuzeville S, Auger JP, Dumesnil A, Roy D, Lacouture S, Fittipaldi N, et al. Serotype-specific role of antigen I/II in the initial steps of the pathogenesis of the infection caused by *Streptococcus suis*. Vet Res. 2017;48(1):39.

47. Du B, Ji W, An H, Shi Y, Huang Q, Cheng Y, et al. Functional analysis of c-di-AMP phosphodiesterase, GdpP, in *Streptococcus suis* serotype 2. Microbiological research. 2014;169(9-10):749-58.

48. Gao T, Tan M, Liu W, Zhang C, Zhang T, Zheng L, et al. GidA, a tRNA Modification Enzyme, Contributes to the Growth, and Virulence of *Streptococcus suis* Serotype 2. Frontiers in cellular and infection microbiology. 2016;6:44.

49. Huang K, Yuan Z, Li J, Zhang Q, Xu Z, Yan S, et al. Identification and characterisation a surface-associated arginine peptidase in *Streptococcus suis* serotype 2. Microbiological research. 2015;170:168-76.

50. Li M, Shao ZQ, Guo Y, Wang L, Hou T, Hu D, et al. The type II histidine triad protein HtpsC is a novel adhesion with the involvement of *Streptococcus suis* virulence. Virulence. 2015;6(6):631-41.

51. Musyoki AM, Shi Z, Xuan C, Lu G, Qi J, Gao F, et al. Structural and functional analysis of an anchorless fibronectin-binding protein FBPS from Gram-positive bacterium *Streptococcus suis*. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(48):13869-74.

52. Shao J, Zhang W, Wu Z, Lu C. The truncated major pilin subunit Sbp2 of the srtBCD pilus cluster still contributes to *Streptococcus suis* pathogenesis in the absence of pilus shaft. Current microbiology. 2014;69(5):703-7.

53. Xiao G, Tang H, Zhang S, Ren H, Dai J, Lai L, et al. *Streptococcus suis* small RNA rss04 contributes to the induction of meningitis by regulating capsule synthesis and by inducing biofilm formation in a mouse infection model. Veterinary microbiology. 2017;199:111-9.

54. Xu J, Fu S, Liu M, Xu Q, Bei W, Chen H, et al. The two-component system NisK/NisR contributes to the virulence of *Streptococcus suis* serotype 2. Microbiological research. 2014;169(7-8):541-6.

55. Yu Y, Qian Y, Du D, Xu C, Dai C, Li Q, et al. SBP2 plays an important role in the virulence changes of different artificial mutants of *Streptococcus suis*. Molecular bioSystems. 2016;12(6):1948-62.

56. Zhang C, Sun W, Tan M, Dong M, Liu W, Gao T, et al. The Eukaryote-Like Serine/Threonine Kinase STK Regulates the Growth and Metabolism of Zoonotic *Streptococcus suis*. Frontiers in cellular and infection microbiology. 2017;7:66.

57. Zhang H, Ma Z, Li Y, Zheng J, Yi L, Fan H, et al. Identification of a novel collagen type capital I, Ukrainian-binding protein from *Streptococcus suis* serotype 2. Veterinary journal. 2013;197(2):406-14.

58. Zhang H, Ravcheev DA, Hu D, Zhang F, Gong X, Hao L, et al. Two novel regulators of N-acetyl-galactosamine utilization pathway and distinct roles in bacterial infections. MicrobiologyOpen. 2015;4(6):983-1000.

59. Zhou J, Zhang X, He K, Wang W, Ni Y, Zhu H, et al. Characterization and proteome analysis of inosine 5-monophosphate dehydrogenase in epidemic Streptococcus suis serotype 2. Current microbiology. 2014;68(5):663-9.

60. Zhu H, Zhou J, Ni Y, Yu Z, Mao A, Hu Y, et al. Contribution of eukaryotic-type serine/threonine kinase to stress response and virulence of *Streptococcus suis*. PLoS One. 2014;9(3):e91971.

61. Zhu J, Zhang T, Su Z, Li L, Wang D, Xiao R, et al. (p)ppGpp synthetases regulate the pathogenesis of zoonotic *Streptococcus suis*. Microbiological research. 2016;191:1-11.

62. Gottschalk M, Segura M. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet Microbiol. 2000;76(3):259-72.

63. Segura M, Fittipaldi N, Calzas C, Gottschalk M. Critical *Streptococcus suis* virulence factors: Are they all really critical? Trends Microbiol. 2017;25(7):585-99.

64. Tenenbaum T, Adam R, Eggelnpohler I, Matalon D, Seibt A, GE KN, et al. Strain-dependent disruption of blood-cerebrospinal fluid barrier by *Streptoccocus suis in vitro*. FEMS Immunol Med Microbiol. 2005;44(1):25-34.

65. Chabot-Roy G, Willson P, Segura M, Lacouture S, Gottschalk M. Phagocytosis and killing of *Streptococcus suis* by porcine neutrophils. Microb Pathog. 2006;41(1):21-32.

66. Benga L, Fulde M, Neis C, Goethe R, Valentin-Weigand P. Polysaccharide capsule and suilysin contribute to extracellular survival of *Streptococcus suis* co-cultivated with primary porcine phagocytes. Vet Microbiol. 2008;132(1-2):211-9.

67. Smith HE, Damman M, van der Velde J, Wagenaar F, Wisselink HJ, Stockhofe-Zurwieden N, et al. Identification and characterization of the cps locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect Immun. 1999;67(4):1750-6.

68. Lecours MP, Fittipaldi N, Takamatsu D, Okura M, Segura M, Goyette-Desjardins G, et al. Sialylation of *Streptococcus suis* serotype 2 is essential for capsule expression but is not responsible for the main capsular epitope. Microbes Infect. 2012;14(11):941-50.

69. Tanabe S, Gottschalk M, Grenier D. Hemoglobin and Streptococcus suis cell wall act in synergy to potentiate the inflammatory response of monocyte-derived macrophages. Innate Immun. 2008;14(6):357-63.

70. Tsiotou AG, Sakorafas GH, Anagnostopoulos G, Bramis J. Septic shock; current pathogenetic concepts from a clinical perspective. Med Sci Monit. 2005;11(3):Ra76-85.

71. Segura M, Gottschalk M. *Streptococcus suis* interactions with the murine macrophage cell line J774: adhesion and cytotoxicity. Infect Immun. 2002;70(8):4312-22.

72. Segura M, Gottschalk M, Olivier M. Encapsulated Streptococcus suis inhibits activation of signaling pathways involved in phagocytosis. Infect Immun. 2004;72(9):5322-30.

73. Segura M, Stankova J, Gottschalk M. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect Immun. 1999;67(9):4646-54.

74. Segura M, Vadeboncoeur N, Gottschalk M. CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by *Streptococcus suis* capsular type 2. Clin Exp Immunol. 2002;127(2):243-54.

75. Zheng H, Ye C, Segura M, Gottschalk M, Xu J. Mitogenic effect contributes to increased virulence of Streptococcus suis sequence type 7 to cause streptococcal toxic shock-like syndrome. Clin Exp Immunol. 2008;153(3):385-91.

76. Dominguez-Punaro M, Segura M, Plante MM, Lacouture S, Rivest S, Gottschalk M. *Streptococcus suis* serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection. J Immunol. 2007;179(3):1842-54.

77. Dominguez-Punaro M, Segura M, Radzioch D, Rivest S, Gottschalk M. Comparison of the susceptibilities of C57BL/6 and A/J mouse strains to *Streptococcus suis* serotype 2 infection. Infect Immun. 2008;76(9):3901-10.

78. Chen C, Tang J, Dong W, Wang C, Feng Y, Wang J, et al. A glimpse of streptococcal toxic shock syndrome from comparative genomics of *Streptococcus suis* 2 Chinese isolates. PLoS One. 2007;2(3):e315.

79. Liu M, Fang L, Tan C, Long T, Chen H, Xiao S. Understanding *Streptococcus suis* serotype 2 infection in pigs through a transcriptional approach. BMC Genomics. 2011;12:253.

80. Wichgers Schreur PJ, Rebel JM, Smits MA, van Putten JP, Smith HE. Differential activation of the Toll-like receptor 2/6 complex by lipoproteins of *Streptococcus suis* serotypes 2 and 9. Vet Microbiol. 2010;143(2-4):363-70.

81. Wichgers Schreur PJ, Rebel JMJ, Smits MA, van Putten JPM, Smith HE. Lgt processing is an essential step in *Streptococcus suis* lipoprotein mediated innate immune activation. PLoS One. 2011;6(7):e22299.

82. Kim KS. Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury. Nat Rev Neurosci. 2003;4(5):376-85.

83. Nassif X, Bourdoulous S, Eugène E, Couraud P. How do extracellular pathogens cross the blood–brain barrier? Trends Microbiol. 2002;10(5):227-32.

84. Dando SJ, Mackay-Sim A, Norton R, Currie BJ, St John JA, Ekberg JA, et al. Pathogens penetrating the central nervous system: infection pathways and the cellular and molecular mechanisms of invasion. Clin Microbiol Rev. 2014;27(4):691-726.

85. Vanier G, Segura M, Friedl P, Lacouture S, Gottschalk M. Invasion of porcine brain microvascular endothelial cells by Streptococcus suis serotype 2. Infect Immun. 2004;72(3):1441-9.

86. Sun Y, Li N, Zhang J, Liu H, Liu J, Xia X, et al. Enolase of *Streptococcus Suis* Serotype 2 Enhances Blood-Brain Barrier Permeability by Inducing IL-8 Release. Inflammation. 2016;39(2):718-26.

87. Wraith DC, Nicholson LB. The adaptive immune system in diseases of the central nervous system. The Journal of Clinical Investigation. 2012;122(4):1172-9.

88. Williams AE, Blakemore WF. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. J Infect Dis. 1990;162(2):474-81.

89. Tenenbaum T, Matalon D, Adam R, Seibt A, Wewer C, Schwerk C, et al. Dexamethasone prevents alteration of tight junction-associated proteins and barrier function in porcine choroid plexus epithelial cells after infection with *Streptococcus suis* in vitro. Brain research. 2008;1229:1-17.

90. Schwerk C, Papandreou T, Schuhmann D, Nickol L, Borkowski J, Steinmann U, et al. Polar invasion and translocation of *Neisseria meningitidis* and *Streptococcus suis* in a novel human model of the blood-cerebrospinal fluid barrier. PLoS One. 2012;7(1):e30069.

91. Tenenbaum T, Papandreou T, Gellrich D, Friedrichs U, Seibt A, Adam R, et al. Polar bacterial invasion and translocation of *Streptococcus suis* across the blood-cerebrospinal fluid barrier in vitro. Cell Microbiol. 2009;11(2):323-36.

92. Schwerk C, Adam R, Borkowski J, Schneider H, Klenk M, Zink S, et al. *In vitro* transcriptome analysis of porcine choroid plexus epithelial cells in response to *Streptococcus suis*: release of pro-inflammatory cytokines and chemokines. Microbes and infection / Institut Pasteur. 2011;13(11):953-62.

93. Gray LD, Fedorko DP. Laboratory diagnosis of bacterial meningitis. Clin Microbiol Rev. 1992;5(2):130-45.

94. Ransohoff RM, Brown MA. Innate immunity in the central nervous system. J Clin Invest. 2012;122(4):1164-71.

95. Seele J, Tauber SC, Bunkowski S, Baums CG, Valentin-Weigand P, de Buhr N, et al. The inflammatory response and neuronal injury in *Streptococcus suis* meningitis. BMC Infect Dis. 2018;18(1):297.

96. Dominguez-Punaro M, Segura M, Contreras I, Lachance C, Houde M, Lecours MP, et al. *In vitro* characterization of the microglial inflammatory response to *Streptococcus suis*, an important emerging zoonotic agent of meningitis. Infect Immun. 2010;78(12):5074-85.

97. Zheng H, Punaro MC, Segura M, Lachance C, Rivest S, Xu J, et al. Toll-like receptor 2 is partially involved in the activation of murine astrocytes by Streptococcus suis, an important zoonotic agent of meningitis. J Neuroimmunol. 2011;234(1-2):71-83.

98. Segura MA, Cleroux P, Gottschalk M. *Streptococcus suis* and Group B *Streptococcus* differ in their interactions with murine macrophages. FEMS Immunol Med Microbiol. 1998;21(3):189-95.

99. Segura M, Fittipaldi N, Calzas C, Gottschalk M. Critical *Streptococcus suis* Virulence Factors: Are They All Really Critical? Trends Microbiol. 2017.

100. Charland N, Harel J, Kobisch M, Lacasse S, Gottschalk M. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology. 1998;144(2):325-32.

101. Roy D, Auger JP, Segura M, Fittipaldi N, Takamatsu D, Okura M, et al. Role of the capsular polysaccharide as a virulence factor for *Streptococcus suis* serotype 14. Can J Vet Res. 2015;79(2):141-6.

102. Feng Y, Cao M, Shi J, Zhang H, Hu D, Zhu J, et al. Attenuation of *Streptococcus suis* virulence by the alteration of bacterial surface architecture. Sci Rep. 2012;2:710.

103. Benga L, Goethe R, Rohde M, Valentin-Weigand P. Non-encapsulated strains reveal novel insights in invasion and survival of *Streptococcus suis* in epithelial cells. Cell Microbiol. 2004;6(9):867-81.

104. Lalonde M, Segura M, Lacouture S, Gottschalk M. Interactions between *Streptococcus suis* serotype 2 and different epithelial cell lines. Microbiology. 2000;146(8):1913-21.

105. Houde M, Gottschalk M, Gagnon F, Van Calsteren MR, Segura M. *Streptococcus suis* capsular polysaccharide inhibits phagocytosis through destabilization of lipid microdomains and prevents lactosylceramide-dependent recognition. Infect Immun. 2012;80(2):506-17.

106. Lecours MP, Segura M, Lachance C, Mussa T, Surprenant C, Montoya M, et al. Characterization of porcine dendritic cell response to *Streptococcus suis*. Vet Res. 2011;42(1):72.

107. Lecours MP, Gottschalk M, Houde M, Lemire P, Fittipaldi N, Segura M. Critical role for *Streptococcus suis* cell wall modifications and suilysin in resistance to complement-dependent killing by dendritic cells. J Infect Dis. 2011;204(6):919-29.

108. Meijerink M, Ferrando ML, Lammers G, Taverne N, Smith HE, Wells JM. Immunomodulatory Effects of Streptococcus suis Capsule Type on Human Dendritic Cell Responses, Phagocytosis and Intracellular Survival. PLoS One. 2012;7(4):e35849.

109. Graveline R, Segura M, Radzioch D, Gottschalk M. TLR2-dependent recognition of Streptococcus suis is modulated by the presence of capsular polysaccharide which modifies macrophage responsiveness. Int Immunol. 2007;19(4):375-89.

110. Gottschalk MG, Lacouture S, Dubreuil JD. Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiology. 1995;141(1):189-95.

111. Palmer M. The family of thiol-activated, cholesterol-binding cytolysins. Toxicon. 2001;39(11):1681-9.

112. Billington SJ, Jost BH, Songer JG. Thiol-activated cytolysins: Structure, function and role in pathogenesis. FEMS Microbiol Lett. 2000;182(2):197-205.

113. Gottschalk MG, Lacouture S, Dubreuil JD. Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiology (Reading, England). 1995;141 (Pt 1):189-95.

114. Tenenbaum T, Seitz M, Schroten H, Schwerk C. Biological activities of suilysin: role in *Streptococcus suis* pathogenesis. Future Microbiol. 2016;11:941-54.

115. Charland N, Nizet V, Rubens CE, Kim KS, Lacouture S, Gottschalk M. *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. Infect Immun. 2000;68(2):637-43.

116. Norton PM, Rolph C, Ward PN, Bentley RW, Leigh JA. Epithelial invasion and cell lysis by virulent strains of *Streptococcus suis* is enhanced by the presence of suilysin. FEMS Immunol Med Microbiol. 1999;26(1):25-35.

117. Vadeboncoeur N, Segura M, Al-Numani D, Vanier G, Gottschalk M. Pro-infammatory cytokine and chemokine release by human brain microvascular endothelial cells stimulated by *Streptococcus suis* serotype 2. FEMS Immunol Med Microbiol. 2003;35(1):49-58.

118. Vanier G, Segura M, Lecours MP, Grenier D, Gottschalk M. Porcine brain microvascular endothelial cell-derived interleukin-8 is first induced and then degraded by Streptococcus suis. Microb Pathog. 2009;46(3):135-43.

119. Segura M, Vanier G, Al-Numani D, Lacouture S, Olivier M, Gottschalk M. Proinflammatory cytokine and chemokine modulation by Streptococcus suis in a whole-blood culture system. FEMS Immunol Med Microbiol. 2006;47(1):92-106.

120. Lun S, Perez-Casal J, Connor W, Willson PJ. Role of suilysin in pathogenesis of *Streptococcus suis* capsular serotype 2. Microb Pathog. 2003;34(1):27-37.

121. Al-Numani D, Segura M, Dore M, Gottschalk M. Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes stimulated by Streptococcus suis serotype 2. Clin Exp Immunol. 2003;133(1):67-77.

122. Jobin MC, Fortin J, Willson PJ, Gottschalk M, Grenier D. Acquisition of plasmin activity and induction of arachidonic acid release by Streptococcus suis in contact with human brain microvascular endothelial cells. FEMS Microbiol Lett. 2005;252(1):105-11.

123. Allen AG, Bolitho S, Lindsay H, Khan S, Bryant C, Norton P, et al. Generation and characterization of a defined mutant of *Streptococcus suis* lacking suilysin. Infect Immun. 2001;69(4):2732-5.

124. Vecht U, Wisselink HJ, Jellema ML, Smith HE. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect Immun. 1991;59(9):3156-62.

125. Vecht U, Arends JP, van der Molen EJ, van Leengoed LA. Differences in virulence between two strains of *Streptococcus suis* type II after experimentally induced infection of newborn germ-free pigs. Am J Vet Res. 1989;50(7):1037-43.

126. Vecht U, Wisselink J, van Dijk JE, Smith HE. Virulence of *Streptococcus suis* type 2 strains in newborn germfree pigs depends on phenotype. Infect Immun. 1992;60(2):550-6.

127. Smith HE, Vecht U, Wisselink HJ, Stockhoff-Zurwieden N, Biermann Y, Smits MA. Mutants of *Streptococcus suis* types 1 and 2 impaired in expression of muramidase-released protein and extracellular protein induce disease in newborn germfree pigs. Infect Immun. 1996;64(10):4409-12.

128. Varela NP, Gadbois P, Thibault C, Gottschalk M, Dick P, Wilson J. Antimicrobial resistance and prudent drug use for *Streptococcus suis*. Anim Health Res Rev. 2013;14(1):68-77.

129. Li LL, Liao XP, Sun J, Yang YR, Liu BT, Yang SS, et al. Antimicrobial resistance, serotypes, and virulence factors of *Streptococcus suis* isolates from diseased pigs. Foodborne Pathog Dis. 2012;9(7):583-8.

130. Cromwell GL. Why and how antibiotics are used in swine production. Anim Biotechnol. 2002;13(1):7-27.

131. Palmieri C, Varaldo PE, Facinelli B. Streptococcus suis, an Emerging Drug-Resistant Animal and Human Pathogen. Front Microbiol. 2011;2:235.

132. Maron DF, Smith TJ, Nachman KE. Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. Globalization and Health. 2013;9(1):48.

133. Segura M. *Streptococcus suis* vaccines: candidate antigens and progress. Expert Rev Vaccines. 2015;14(12):1587-608.

134. Calzas C, Goyette-Desjardins G, Lemire P, Gagnon F, Lachance C, Van Calsteren MR, et al. Group B *Streptococcus* and *Streptococcus suis* capsular polysaccharides induce chemokine production by dendritic cells via Toll-like receptor 2- and MyD88-dependent and -independent pathways. Infect Immun. 2013;81(9):3106-18.

135. Calzas C, Lemire P, Auray G, Gerdts V, Gottschalk M, Segura M. Antibody response specific to the capsular polysaccharide is impaired in *Streptococcus suis* serotype 2-infected animals. Infect Immun. 2015;83(1):441-53.

136. Letendre C, Auger JP, Lemire P, Galbas T, Gottschalk M, Thibodeau J, et al. Streptococcus suis Serotype 2 Infection Impairs Interleukin-12 Production and the MHC-II-Restricted Antigen Presentation Capacity of Dendritic Cells. Front Immunol. 2018;9:1199.

137. Lecours MP, Letendre C, Clarke D, Lemire P, Galbas T, Benoit-Biancamano MO, et al. Immune-responsiveness of CD4+ T cells during *Streptococcus suis* serotype 2 infection. Sci Rep. 2016;6:38061.

138. Pallares FJ, Schmitt CS, Roth JA, Evans RB, Kinyon JM, Halbur PG. Evaluation of a ceftiofur-washed whole cell Streptococcus suis bacterin in pigs. Can J Vet Res. 2004;68(3):236-40.

139. Esgleas M, Li Y, Hancock MA, Harel J, Dubreuil JD, Gottschalk M. Isolation and characterization of alpha-enolase, a novel fibronectin-binding protein from *Streptococcus suis*. Microbiology. 2008;154(Pt 9):2668-79.

140. Esgleas M, Dominguez-Punaro MC, Li Y, Harel J, Dubreuil JD, Gottschalk M. Immunization with SsEno fails to protect mice against challenge with *Streptococcus suis* serotype 2. FEMS Microbiol Lett. 2009;294(1):82-8.

141. Zhang A, Chen B, Mu X, Li R, Zheng P, Zhao Y, et al. Identification and characterization of a novel protective antigen, Enolase of Streptococcus suis serotype 2. Vaccine. 2009;27(9):1348-53.

142. Feng Y, Pan X, Sun W, Wang C, Zhang H, Li X, et al. Streptococcus suis enolase functions as a protective antigen displayed on the bacterial cell surface. J Infect Dis. 2009;200(10):1583-92.

143. Li Y, Martinez G, Gottschalk M, Lacouture S, Willson P, Dubreuil JD, et al. Identification of a surface protein of *Streptococcus suis* and evaluation of its immunogenic and protective capacity in pigs. Infect Immun. 2006;74(1):305-12.

144. Li Y, Gottschalk M, Esgleas M, Lacouture S, Dubreuil JD, Willson P, et al. Immunization with Recombinant Sao Protein Confers Protection against Streptococcus suis Infection. Clin Vaccine Immunol. 2007;14(8):937-43.

145. Garibaldi M, Rodriguez-Ortega MJ, Mandanici F, Cardaci A, Midiri A, Papasergi S, et al. Immunoprotective activities of a Streptococcus suis pilus subunit in murine models of infection. Vaccine. 2010;28(20):3609-16.

146. Mandanici F, Gomez-Gascon L, Garibaldi M, Olaya-Abril A, Luque I, Tarradas C, et al. A surface protein of Streptococcus suis serotype 2 identified by proteomics protects mice against infection. J Proteomics. 2010;73(12):2365-9.

147. Hsueh KJ, Lee JW, Hou SM, Chen HS, Chang TC, Chu CY. Evaluation on a Streptococcus suis vaccine using recombinant sao-l protein manufactured by bioreactors as the antigen in pigs. Transbound Emerg Dis. 2014;61(6):e35-43.

148. Roy D, Fittipaldi N, Dumesnil A, Lacouture S, Gottschalk M. The protective protein Sao (surface antigen one) is not a critical virulence factor for Streptococcus suis serotype 2. Microb Pathog. 2014;67-68:31-5.

149. Song JY, Moseley MA, Burton RL, Nahm MH. Pneumococcal vaccine and opsonic pneumococcal antibody. J Infect Chemother. 2013;19(3):412-25.

150. Heath PT. Status of vaccine research and development of vaccines for GBS. Vaccine. 2016;34(26):2876-9.

151. Bottomley MJ, Serruto D, Sáfadi MAP, Klugman KP. Future challenges in the elimination of bacterial meningitis. Vaccine. 2012;30(Supplement 2):B78-B86.

152. Jones C. Vaccines based on the cell surface carbohydrates of pathogenic bacteria. An Acad Bras Cienc. 2005;77:293-324.

153. Avci FY, Kasper DL. How Bacterial Carbohydrates Influence the Adaptive Immune System. Annu Rev Immunol. 2010;28(1):107-30.

154. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. Biochem Cell Biol. 2010;88(3):513-25.

155. Varki A, Cummings RD, Aebi M, Packer NH, Seeberger PH, Esko JD, et al. Symbol Nomenclature for Graphical Representations of Glycans. Glycobiology. 2015;25(12):1323-4.

156. Smith HE, Damman M, van der Velde J, Wagenaar F, Wisselink HJ, Stockhofe-Zurwieden N, et al. Identification and characterization of the cps locus of Streptococcus suis serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infection and immunity. 1999;67(4):1750-6.

157. Okura M, Takamatsu D, Maruyama F, Nozawa T, Nakagawa I, Osaki M, et al. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: potential mechanisms for generation of capsular variation. Appl Environ Microbiol. 2013;79(8):2796-806.

158. Charland N, Kellens JT, Caya F, Gottschalk M. Agglutination of *Streptococcus suis* by sialic acid-binding lectins. J Clin Microbiol. 1995;33(8):2220-1.

159. Shibuya N, Goldstein IJ, Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ. The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. J Biol Chem. 1987;262(4):1596-601.

160. Pan Z, Ma J, Dong W, Song W, Wang K, Lu C, et al. Novel variant serotype of streptococcus suis isolated from piglets with meningitis. Applied and environmental microbiology. 2015;81(3):976-85.

161. Qiu X, Bai X, Lan R, Zheng H, Xu J. Novel Capsular Polysaccharide Loci and New Diagnostic Tools for High-Throughput Capsular Gene Typing in Streptococcus suis. Applied and environmental microbiology. 2016;82(24):7102-12.

162. Zhang Y, Ding D, Liu M, Yang X, Zong B, Wang X, et al. Effect of the glycosyltransferases on the capsular polysaccharide synthesis of *Streptococcus suis* serotype 2. Microbiol Res. 2016;185:45-54.

163. Yother J. Capsules of Streptococcus pneumoniae and other bacteria: paradigms for polysaccharide biosynthesis and regulation. Annual review of microbiology. 2011;65:563-81.

164. Lakkitjaroen N, Takamatsu D, Okura M, Sato M, Osaki M, Sekizaki T. Capsule loss or death: the position of mutations among capsule genes sways the destiny of Streptococcus suis. FEMS microbiology letters. 2014;354(1):46-54.

165. Dzanibe S, Madhi SA. Systematic review of the clinical development of group B streptococcus serotype-specific capsular polysaccharide-based vaccines. Expert Rev Vaccines. 2018;17(7):635-51.

166. Zou W, Mackenzie R, Therien L, Hirama T, Yang Q, Gidney MA, et al. Conformational epitope of the type III group B Streptococcus capsular polysaccharide. J Immunol. 1999;163(2):820-5.

167. Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, et al. Structural and genetic diversity of group B streptococcus capsular polysaccharides. Infection and immunity. 2005;73(5):3096-103.

168. Chaffin DO, Mentele LM, Rubens CE. Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. Journal of bacteriology. 2005;187(13):4615-26.

169. Segura M. Fisher scientific award lecture - The capsular polysaccharides of Group B *Streptococcus* and *Streptococcus suis* differently modulate bacterial interactions with dendritic cells. Can J Microbiol. 2012;58(3):249-60.

170. Segura MA, Cleroux P, Gottschalk M. Streptococcus suis and group B Streptococcus differ in their interactions with murine macrophages. FEMS immunology and medical microbiology. 1998;21(3):189-95.

171. Jennings HJ, Katzenellenbogen E, Lugowski C, Kasper DL. Structure of native polysaccharide antigens of type Ia and type Ib Group B *Streptococcus*. Biochemistry. 1983;22(5):1258-64.

172. Jennings HJ, Rosell KG, Katzenellenbogen E, Kasper DL. Structural determination of the capsular polysaccharide antigen of type II Group B *Streptococcus*. J Biol Chem. 1983;258(3):1793-8.

173. Jennings HJ, Lugowski C, Kasper DL. Conformational aspects critical to the immunospecificity of the type III Group B streptococcal polysaccharide. Biochemistry. 1981;20(16):4511-8.

174. Wessels MR, Pozsgay V, Kasper DL, Jennings HJ. Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III Group B *Streptococcus*. A revised structure for the type III Group B streptococcal polysaccharide antigen. J Biol Chem. 1987;262(17):8262-7.

175. Di Fabio JL, Michon F, Brisson J-R, Jennings HJ, Wessels MR, Benedí V-J, et al. Structure of the capsular polysaccharide antigen of type IV Group B *Streptococcus*. Can J Chem. 1989;67(5):877-82.

176. Wessels MR, DiFabio JL, Benedi VJ, Kasper DL, Michon F, Brisson JR, et al. Structural determination and immunochemical characterization of the type V Group B *Streptococcus* capsular polysaccharide. J Biol Chem. 1991;266(11):6714-9.

177. von Hunolstein C, D'Ascenzi S, Wagner B, Jelinkova J, Alfarone G, Recchia S, et al. Immunochemistry of capsular type polysaccharide and virulence properties of type VI *Streptococcus agalactiae* (Group B streptococci). Infect Immun. 1993;61(4):1272-80.

178. Kogan G, Brisson JR, Kasper DL, von Hunolstein C, Orefici G, Jennings HJ. Structural elucidation of the novel type VII Group B *Streptococcus* capsular polysaccharide by high resolution NMR spectroscopy. Carbohydr Res. 1995;277(1):1-9.

179. Kogan G, Uhrin D, Brisson JR, Paoletti LC, Blodgett AE, Kasper DL, et al. Structural and immunochemical characterization of the type VIII Group B *Streptococcus* capsular polysaccharide. J Biol Chem. 1996;271(15):8786-90.

180. Berti F, Campisi E, Toniolo C, Morelli L, Crotti S, Rosini R, et al. Structure of the type IX Group B *Streptococcus* capsular polysaccharide and its evolutionary relationship with types V and VII. J Biol Chem. 2014;289(34):23437-48.

181. Mook-Kanamori BB, Geldhoff M, van der Poll T, van de Beek D. Pathogenesis and pathophysiology of pneumococcal meningitis. Clin Microbiol Rev. 2011;24(3):557-91.

182. Simell B, Auranen K, Käyhty H, Goldblatt D, Dagan R, O'Brien KL. The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines. 2012;11(7):841-55.

183. Henriques-Normark B, Tuomanen EI. The *Pneumococcus*: epidemiology, microbiology, and pathogenesis. Cold Spring Harb Perspect Med. 2013;3(7).

184. Nuorti JP, Whitney CG. Prevention of pneumococcal disease among infants and children - use of 13-valent pneumococcal conjugate vaccine and 23-valent pneumococcal polysaccharide vaccine - recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2010;59(Rr-11):1-18.

185. Geno KA, Gilbert GL, Song JY, Skovsted IC, Klugman KP, Jones C, et al. Pneumococcal capsules and their types: past, present, and future. Clin Microbiol Rev. 2015;28(3):871-99.

186. Daniels CC, Rogers PD, Shelton CM. A Review of Pneumococcal Vaccines: Current Polysaccharide Vaccine Recommendations and Future Protein Antigens. The journal of pediatric pharmacology and therapeutics : JPPT : the official journal of PPAG. 2016;21(1):27-35.

187. Yother J. Capsules of *Streptococcus pneumoniae* and Other Bacteria: Paradigms for Polysaccharide Biosynthesis and Regulation. Annu Rev Microbiol. 2011;65(1):563-81.

188. Nelson AL, Roche AM, Gould JM, Chim K, Ratner AJ, Weiser JN. Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. Infect Immun. 2007;75(1):83-90.

189. Stephen TL, Groneck L, Kalka-Moll WM. The modulation of Adaptative Immune Responses by Bacterial Zwitterionic Polysaccharides. International Journal of Microbiology. 2010;2010:12.

190. Kim JO, Romero-Steiner S, Sorensen UB, Blom J, Carvalho M, Barnard S, et al. Relationship between cell surface carbohydrates and intrastrain variation on opsonophagocytosis of Streptococcus pneumoniae. Infect Immun. 1999;67(5):2327-33.

191. Abbas AK, Lichtman AH, Pillai S. Cellular and Molecular Immunology. 8th ed. Philadelphia, PA, USA: Elsevier/Saunders; 2015. 535 p.

192. De Nardo D. Toll-like receptors: Activation, signalling and transcriptional modulation. Cytokine. 2015;74(2):181-9.

193. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol. 2014;5:461.

194. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34(5):637-50.

195. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805-20.

196. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology. 2010;11(5):373-84.

197. Bi L, Pian Y, Chen S, Ren Z, Liu P, Lv Q, et al. Toll-like receptor 4 confers inflammatory response to suilysin. Frontiers in microbiology. 2015;6:644.

198. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4(7):499-511.

199. Jimenez-Dalmaroni MJ, Gerswhin ME, Adamopoulos IE. The critical role of toll-like receptors--From microbial recognition to autoimmunity: A comprehensive review. Autoimmunity reviews. 2016;15(1):1-8.

200. Vignali DA, Kuchroo VK. IL-12 family cytokines: immunological playmakers. Nat Immunol. 2012;13(8):722-8.

201. Romagnani S. Type 1 T helper and type 2 T helper cells: functions, regulation and role in protection and disease. Int J Clin Lab Res. 1991;21(2):152-8.

202. Shibuya K, Robinson D, Zonin F, Hartley SB, Macatonia SE, Somoza C, et al. IL-1 alpha and TNF-alpha are required for IL-12-induced development of Th1 cells producing high levels of IFN-gamma in BALB/c but not C57BL/6 mice. J Immunol. 1998;160(4):1708-16.

203. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta. 2011;1813(5):878-88.

204. Koch F, Stanzl U, Jennewein P, Janke K, Heufler C, Kampgen E, et al. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. J Exp Med. 1996;184(2):741-6.

205. Gatto D, Brink R. The germinal center reaction. J Allergy Clin Immunol. 2010;126(5):898-907.

206. Mond JJ, Lees A, Snapper CM. T Cell-Independent Antigens Type 2. Annu Rev Immunol. 1995;13(1):655-92.

207. Snapper CM. Differential regulation of polysaccharide-specific antibody responses to isolated polysaccharides, conjugate vaccines, and intact Gram-positive versus Gram-negative extracellular bacteria. Vaccine. 2016;34(30):3542-8.

208. Avci FY, Li X, Tsuji M, Kasper DL. A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. Nat Med. 2011;17(12):1602-9.

209. Lang ML. The Influence of Invariant Natural Killer T Cells on Humoral Immunity to T-Dependent and -Independent Antigens. Front Immunol. 2018;9(305).

210. Souza-Fonseca-Guimaraes F, Adib-Conquy M, Cavaillon JM. Natural killer (NK) cells in antibacterial innate immunity: angels or devils? Mol Med. 2012;18:270-85.

211. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li X, et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. Nat Immunol. 2011;12(10):966-74.

212. Lemire P, Galbas T, Thibodeau J, Segura M. Natural Killer Cell Functions during the Innate Immune Response to Pathogenic Streptococci. Front Microbiol. 2017;8:1196.

213. Collins AM. IgG subclass co-expression brings harmony to the quartet model of murine IgG function. Immunol Cell Biol. 2016;94(10):949-54.

214. Crawley A, Raymond C, Wilkie BN. Control of immunoglobulin isotype production by porcine B-cells cultured with cytokines. Vet Immunol Immunopathol. 2003;91(2):141-54.

215. Crawley A, Wilkie BN. Porcine Ig isotypes: function and molecular characteristics. Vaccine. 2003;21(21–22):2911-22.

216. Butler JE, Wertz N, Deschacht N, Kacskovics I. Porcine IgG: structure, genetics, and evolution. Immunogenetics. 2009;61(3):209-30.

217. Baums CG, Brüggemann C, Kock C, Beineke A, Waldmann K-H, Valentin-Weigand P. Immunogenicity of an autogenous *Streptococcus suis* bacterin in preparturient sows and their piglets in relation to protection after weaning. Clin Vaccine Immunol. 2010;17(10):1589-97.

218. Guilliams M, Bruhns P, Saeys Y, Hammad H, Lambrecht BN. The function of Fcγ receptors in dendritic cells and macrophages. Nat Rev Immunol. 2014;14(2):94-108.

219. Michaelsen TE, Kolberg J, Aase A, Herstad TK, Høiby EA. The four mouse IgG isotypes differ extensively in bactericidal and opsonophagocytic activity when reacting with the P1.16 epitope on the outer membrane PorA protein of *Neisseria meningitidis*. Scand J Immunol. 2004;59(1):34-9.

220. Goh YS, Grant AJ, Restif O, McKinley TJ, Armour KL, Clark MR, et al. Human IgG isotypes and activating Fcgamma receptors in the interaction of Salmonella enterica serovar Typhimurium with phagocytic cells. Immunology. 2011;133(1):74-83.

221. Roy D, Grenier D, Segura M, Mathieu-Denoncourt A, Gottschalk M. Recruitment of Factor H to the Streptococcus suis Cell Surface is Multifactorial. Pathogens. 2016;5(3).

222. Wibroe PP, Helvig SY, Moein Moghimi S. The role of complement in antibody therapy for infectious diseases. Microbiol Spectr. 2014;2(2).

223. Macleod CM, Hodges RG, Heidelberger M, Bernhard WG. PREVENTION OF PNEUMOCOCCAL PNEUMONIA BY IMMUNIZATION WITH SPECIFIC CAPSULAR POLYSACCHARIDES. J Exp Med. 1945;82(6):445-65.

224. Micoli F, Adamo R, Costantino P. Protein Carriers for Glycoconjugate Vaccines: History, Selection Criteria, Characterization and New Trends. Molecules (Basel, Switzerland). 2018;23(6):1451.

225. Micoli F, Costantino P, Adamo R. Potential targets for next generation anti-microbial glycoconjugate vaccines. FEMS Microbiol Rev. 2018.

226. Verez-Bencomo V, Fernandez-Santana V, Hardy E, Toledo ME, Rodriguez MC, Heynngnezz L, et al. A synthetic conjugate polysaccharide vaccine against Haemophilus influenzae type b. Science. 2004;305(5683):522-5.

227. Fernàndez-Santana V, Cardoso F, Rodriguez A, Carmenate T, Pena L, Valdés Y, et al. Antigenicity and Immunogenicity of a Synthetic Oligosaccharide-Protein Conjugate Vaccine against *Haemophilus influenzae* Type b. Infect Immun. 2004;72(12):7115-23.

228. Gerdts V, Mutwiri G, Richards J, Hurk SvDL-vd, Potter AA. Carrier molecules for use in veterinary vaccines. Vaccine. 2013;31(4):596-602.

229. Byrd W, Kadis S. Preparation, characterization, and immunogenicity of conjugate vaccines directed against *Actinobacillus pleuropneumoniae* virulence determinants. Infect Immun. 1992;60(8):3042-51.

230. Byrd W, Harmon BG, Kadis S. Protective efficacy of conjugate vaccines against experimental challenge with porcine *Actinobacillus pleuropneumoniae*. Vet Immunol Immunopathol. 1992;34(3-4):307-24.

231. Andresen LO, Jacobsen MJ, Nielsen JP. Experimental vaccination of pigs with an *Actinobacillus pleuropneumoniae* serotype 5b capsular polysaccharide-tetanus toxoid conjugate. Acta Vet Scand. 1997;38(3):283-93.

232. Knuf M, Kowalzik F, Kieninger D. Comparative effects of carrier proteins on vaccine-induced immune response. Vaccine. 2011;29(31):4881-90.

233. Pichichero ME. Protein carriers of conjugate vaccines. Hum Vaccin Immunother. 2013;9(12):2505-23.

234. Pozsgay V, Kubler-Kielb J. Conjugation Methods toward Synthetic Vaccines. Carbohydrate-Based Vaccines. ACS Symposium Series. 989: American Chemical Society; 2008. p. 36-70.

235. Farkaš P, Bystrický S. Chemical conjugation of biomacromolecules: A mini-review. Chemical Papers. 2010;64(6):683-95.

236. Hermanson GT. Bioconjugate Techniques. 3rd ed. San Diego, CA, USA: Elsevier; 2013. 1200 p.

237. Pavliakova D, Chu C, Bystricky S, Tolson NW, Shiloach J, Kaufman JB, et al. Treatment with succinic anhydride improves the immunogenicity of Shigella flexneri type 2a O-specific polysaccharide-protein conjugates in mice. Infect Immun. 1999;67(10):5526-9.

238. Reuter G, Schauer R, Szeiki C, Kamerling JP, Vliegenthart JF. A detailed study of the periodate oxidation of sialic acids in glycoproteins. Glycoconj J. 1989;6(1):35-44.

239. Wessels MR, Paoletti LC, Kasper DL, DiFabio JL, Michon F, Holme K, et al. Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against type III Group B *Streptococcus*. J Clin Invest. 1990;86(5):1428-33.

240. Stills HF. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR Journal. 2005;46(3):280-93.

241. Gerdts V. Adjuvants for veterinary vaccines--types and modes of action. Berl Munch Tierarztl Wochenschr. 2015;128(11-12):456-63.

242. Scheiermann J, Klinman DM. Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. Vaccine. 2014;32(48):6377-89.

243. Ming F, Yang J, Chu P, Ma M, Shi J, Cai H, et al. Immunization of Aged Pigs with Attenuated Pseudorabies Virus Vaccine Combined with CpG Oligodeoxynucleotide Restores Defective Th1 Immune Responses. PLoS One. 2013;8(6):e65536.

244. Muzalevskaya EN, Miroshnichenko LA, Nikolaevskii VA, Ushakov IB, Chernov YN, Alabovskii VV, et al. [SQUALENE: PHYSIOLOGICAL AND PHARMACOLOGICAL PROPERTIES]. Eksp Klin Farmakol. 2015;78(6):30-6.

245. Kalvodova L. Squalene-based oil-in-water emulsion adjuvants perturb metabolism of neutral lipids and enhance lipid droplet formation. Biochem Biophys Res Commun. 2010;393(3):350-5.

246. Wilkins AL, Kazmin D, Napolitani G, Clutterbuck EA, Pulendran B, Siegrist CA, et al. AS03- and MF59-Adjuvanted Influenza Vaccines in Children. Front Immunol. 2017;8:1760.

247. Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunol Cell Biol. 2004;82(5):488-96.

248. Aucouturier J, Dupuis L, Ganne V. Adjuvants designed for veterinary and human vaccines. Vaccine. 2001;19(17-19):2666-72.

249. Sun HX, Xie Y, Ye YP. Advances in saponin-based adjuvants. Vaccine. 2009;27(12):1787-96.

250. Rajput ZI, Hu SH, Xiao CW, Arijo AG. Adjuvant effects of saponins on animal immune responses. Journal of Zhejiang University Science B. 2007;8(3):153-61.

251. Ghimire TR. The mechanisms of action of vaccines containing aluminum adjuvants: an in vitro vs in vivo paradigm. SpringerPlus. 2015;4:181.

252. Higgins R, Gottschalk M. Streptococcal diseases. In: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ, editors. Diseases of swine. 9th ed. Ames, USA: Blackwell Publishing; 2006. p. 769-83.

253. Messier S, Lacouture S, Gottschalk M. Distribution of *Streptococcus suis* capsular types from 2001 to 2007. Can Vet J. 2008;49(5):461-2.

254. Gottschalk M, Lacouture S. Canada: Distribution of *Streptococcus suis* (from 2012 to 2014) and *Actinobacillus pleuropneumoniae* (from 2011 to 2014) serotypes isolated from diseased pigs. Can Vet J. 2015;56(10):1093-4.

255. Wisselink HJ, Smith HE, Stockhofe-Zurwieden N, Peperkamp K, Vecht U. Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of *Streptococcus suis* strains isolated from diseased pigs in seven European countries. Vet Microbiol. 2000;74(3):237-48.

256. Vela AI, Goyache J, Tarradas C, Luque I, Mateos A, Moreno MA, et al. Analysis of genetic diversity of *Streptococcus suis* clinical isolates from pigs in Spain by pulsed-field gel electrophoresis. J Clin Microbiol. 2003;41(6):2498-502.

257. Tarradas C, Perea A, Vela AI, Goyache J, Dominguez L, Fernandez-Garaizabal JF, et al. Distribution of serotypes of *Streptococcus suis* isolated from diseased pigs in Spain. Vet Rec. 2004;154(21):665-6.

258. Luque I, Blume V, Borge C, Vela AI, Perea JA, Marquez JM, et al. Genetic analysis of *Streptococcus suis* isolates recovered from diseased and healthy carrier pigs at different stages of production on a pig farm. Vet J. 2010;186(3):396-8.

259. Ngo TH, Tran TB, Tran TT, Nguyen VD, Campbell J, Pham HA, et al. Slaughterhouse pigs are a major reservoir of *Streptococcus suis* serotype 2 capable of causing human infection in southern Vietnam. PLoS One. 2011;6(3):e17943.

260. Kerdsin A, Dejsirilert S, Akeda Y, Sekizaki T, Hamada S, Gottschalk M, et al. Fifteen *Streptococcus suis* serotypes identified by multiplex PCR. J Med Microbiol. 2012;61(Pt 12):1669-72.

261. Padungtod P, Tharavichitkul P, Junya S, Chaisowong W, Kadohira M, Makino S, et al. Incidence and presence of virulence factors of *Streptococcus suis* infection in

slaughtered pigs from Chiang Mai, Thailand. Southeast Asian J Trop Med Public Health. 2010;41(6):1454-61.

262. Hoa NT, Chieu TT, Do Dung S, Long NT, Hieu TQ, Luc NT, et al. *Streptococcus suis* and porcine reproductive and respiratory syndrome, Vietnam. Emerg Infect Dis. 2013;19(2):331-3.

263. Marois C, Le Devendec L, Gottschalk M, Kobisch M. Detection and molecular typing of *Streptococcus suis* in tonsils from live pigs in France. Can J Vet Res. 2007;71(1):14-22.

264. Wang K, Zhang W, Li X, Lu C, Chen J, Fan W, et al. Characterization of *Streptococcus suis* isolates from slaughter swine. Curr Microbiol. 2013;66(4):344-9.

265. Vilaichone RK, Vilaichone W, Nunthapisud P, Wilde H. *Streptococcus suis* infection in Thailand. J Med Assoc Thai. 2002;85:S109-17.

266. Kerdsin A, Akeda Y, Takeuchi D, Dejsirilert S, Gottschalk M, Oishi K. Genotypic diversity of *Streptococcus suis* strains isolated from humans in Thailand. Eur J Clin Microbiol Infect Dis. 2018;37(5):917-25.

267. Callejo R, Zheng H, Du P, Prieto M, Xu J, Zielinski G, et al. *Streptococcus suis* serotype 2 strains isolated in Argentina (South America) are different from those recovered in North America and present a higher risk for humans. JMM case reports. 2016;3(5):e005066.

268. Taniyama D, Sakurai M, Sakai T, Kikuchi T, Takahashi T. Human case of bacteremia due to *Streptococcus suis* serotype 5 in Japan: The first report and literature review. IDCases. 2016;6:36-8.

269. Gustavsson C, Rasmussen M. Septic arthritis caused by *Streptococcus suis* serotype 5 in pig farmer. Emerg Infect Dis. 2014;20(3):489-90.

270. Kerdsin A, Hatrongjit R, Gottschalk M, Takeuchi D, Hamada S, Akeda Y, et al. Emergence of *Streptococcus suis* serotype 9 infection in humans. J Microbiol Immunol Infect. 2017;50(4):545-6.

271. Raberahona M, Rasoanandrasana S, Rahajamanana VL, Ranaivo-Rabetokotany F, Andriananja V, Rakotomalala FA, et al. Novel *Streptococcus suis* Sequence Type 834 among Humans, Madagascar. Emerg Infect Dis. 2018;24(2):391-2.

272. Prince-David M, Salou M, Marois-Crehan C, Assogba K, Plainvert C, Balogou KA, et al. Human meningitis due to *Streptococcus suis* in Lome, Togo: a case report. BMC Infect Dis. 2016;16(1):651.

273. Tall H, Njanpop-Lafourcade BM, Mounkoro D, Tidjani L, Agbenoko K, Alassani I, et al. Identification of *Streptococcus suis* Meningitis through Population-Based Surveillance, Togo, 2010-2014. Emerg Infect Dis. 2016;22(7):1262-4.

274. Gomez-Torres J, Nimir A, Cluett J, Aggarwal A, Elsayed S, Soares D, et al. Human Case of *Streptococcus suis* Disease, Ontario, Canada. Emerg Infect Dis. 2017;23(12):2107-9.

275. Yanase T, Morii D, Kamio S, Nishimura A, Fukao E, Inose Y, et al. The first report of human meningitis and pyogenic ventriculitis caused by *Streptococcus suis*: A case report. J Infect Chemother. 2018.

276. Nutravong T, Angkititrakul S, Jiwakanon N, Wongchanthong W, Dejsirilerts S, Nawa Y. Identification of major *Streptococcus suis* serotypes 2, 7, 8 and 9 isolated from pigs and humans in upper northeastern Thailand. Southeast Asian J Trop Med Public Health. 2014;45(5):1173-81.

277. Praphasiri P, Owusu JT, Thammathitiwat S, Ditsungnoen D, Boonmongkon P, Sangwichian O, et al. *Streptococcus suis* infection in hospitalized patients, Nakhon Phanom Province, Thailand. Emerg Infect Dis. 2015;21(2):345-8.

278. Nguyen BH, Phan DH, Nguyen HX, Le AV, Alberti A. Molecular diagnostics and ITS-based phylogenic analysis of *Streptococcus suis* serotype 2 in central Vietnam. J Infect Dev Ctries. 2015;9(6):624-30.

279. Eisenberg T, Hudemann C, Hossain HM, Hewer A, Tello K, Bandorski D, et al. Characterization of Five Zoonotic *Streptococcus suis* Strains from Germany, Including One Isolate from a Recent Fatal Case of Streptococcal Toxic Shock-Like Syndrome in a Hunter. J Clin Microbiol. 2015;53(12):3912-5.

280. Mancini F, Adamo F, Creti R, Monaco M, Alfarone G, Pantosti A, et al. A fatal case of streptococcal toxic shock syndrome caused by *Streptococcus suis* carrying tet (40) and tet (O/W/32/O), Italy. J Infect Chemother. 2016;22(11):774-6.

281. van Samkar A, Brouwer MC, Schultsz C, van der Ende A, van de Beek D. *Streptococcus suis* meningitis in the Netherlands. J Infect. 2015;71(5):602-4.

282. Bojarska A, Molska E, Janas K, Skoczynska A, Stefaniuk E, Hryniewicz W, et al. *Streptococcus suis* in invasive human infections in Poland: clonality and determinants of virulence and antimicrobial resistance. Eur J Clin Microbiol Infect Dis. 2016;35(6):917-25.

283. Gomez-Zorrilla S, Ardanuy C, Lora-Tamayo J, Camara J, Garcia-Somoza D, Pena C, et al. *Streptococcus suis* infection and malignancy in man, Spain. Emerg Infect Dis. 2014;20(6):1067-8.

284. Fernandez-Aguilar X, Gottschalk M, Aragon V, Camara J, Ardanuy C, Velarde R, et al. Urban Wild Boars and Risk for Zoonotic *Streptococcus suis*, Spain. Emerg Infect Dis. 2018;24(6):1083-6.

285. Mai NT, Hoa NT, Nga TV, Linh le D, Chau TT, Sinh DX, et al. *Streptococcus suis* meningitis in adults in Vietnam. Clin Infect Dis. 2008;46(5):659-67.

286. Suankratay C, Intalapaporn P, Nunthapisud P, Arunyingmongkol K, Wilde H. *Streptococcus suis* meningitis in Thailand. Southeast Asian J Trop Med Public Health. 2004;35(4):868-76.

287. Hui AC, Ng KC, Tong PY, Mok V, Chow KM, Wu A, et al. Bacterial meningitis in Hong Kong: 10-years' experience. Clin Neurol Neurosurg. 2005;107(5):366-70.

288. Varadi L, Luo JL, Hibbs DE, Perry JD, Anderson RJ, Orenga S, et al. Methods for the detection and identification of pathogenic bacteria: past, present, and future. Chem Soc Rev. 2017;46(16):4818-32.

289. Athey TB, Teatero S, Lacouture S, Takamatsu D, Gottschalk M, Fittipaldi N. Determining *Streptococcus suis* serotype from short-read whole-genome sequencing data. BMC Microbiol. 2016;16(1):162.

290. Morrison MJ, Imperiali B. The Renaissance of Bacillosamine and Its Derivatives: Pathway Characterization and Implications in Pathogenicity. Biochemistry. 2014;53(4):624-38.

291. Wang Y, Hollingsworth RI, Kasper DL. Ozonolytic depolymerization of polysaccharides in aqueous solution. Carbohydr Res. 1999;319(1-4):141-7.

292. Charland N, Jacques M, Lacouture S, Gottschalk M. Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by *Streptococcus suis* serotypes 1, 2 and 1/2. Microbiology. 1997;143(11):3607-14.

293. Brisson JR, Uhrinova S, Woods RJ, van der Zwan M, Jarrell HC, Paoletti LC, et al. NMR and molecular dynamics studies of the conformational epitope of the type III group B Streptococcus capsular polysaccharide and derivatives. Biochemistry. 1997;36(11):3278-92.

294. Zou W, Mackenzie R, Thérien L, Hirama T, Yang Q, Gidney MA, et al. Conformational epitope of the type III Group B *Streptococcus* capsular polysaccharide. J Immunol. 1999;163(2):820-5.

295. MacKenzie CR, Jennings HJ. Characterization of polysaccharide conformational epitopes by surface plasmon resonance. Methods Enzymol. 2003;363:340-54.

296. González-Outeiriño J, Kadirvelraj R, Woods RJ. Structural elucidation of type III group B *Streptococcus* capsular polysaccharide using molecular dynamics simulations: the role of sialic acid. Carbohydr Res. 2005;340(5):1007-18.

297. Carboni F, Adamo R, Fabbrini M, De Ricco R, Cattaneo V, Brogioni B, et al. Structure of a protective epitope of group B *Streptococcus* type III capsular polysaccharide. Proc Natl Acad Sci U S A. 2017;114(19):5017-22.

298. Parameswarappa SG, Reppe K, Geissner A, Menova P, Govindan S, Calow ADJ, et al. A Semi-synthetic Oligosaccharide Conjugate Vaccine Candidate Confers Protection against Streptococcus pneumoniae Serotype 3 Infection. Cell chemical biology. 2016;23(11):1407-16.

299. Geissner A, Pereira CL, Leddermann M, Anish C, Seeberger PH. Deciphering Antigenic Determinants of Streptococcus pneumoniae Serotype 4 Capsular Polysaccharide using Synthetic Oligosaccharides. ACS Chem Biol. 2016;11(2):335-44.

300. Lisboa MP, Khan N, Martin C, Xu F-F, Reppe K, Geissner A, et al. Semisynthetic glycoconjugate vaccine candidate against Streptococcus pneumoniae serotype 5. Proc Natl Acad Sci U S A. 2017;114(42):11063-8.

301. Schumann B, Hahm HS, Parameswarappa SG, Reppe K, Wahlbrink A, Govindan S, et al. A semisynthetic Streptococcus pneumoniae serotype 8 glycoconjugate vaccine. Sci Transl Med. 2017;9(380).

302. Emmadi M, Khan N, Lykke L, Reppe K, S GP, Lisboa MP, et al. A Streptococcus pneumoniae Type 2 Oligosaccharide Glycoconjugate Elicits Opsonic Antibodies and Is Protective in an Animal Model of Invasive Pneumococcal Disease. J Am Chem Soc. 2017;139(41):14783-91.

303. Schumann B, Reppe K, Kaplonek P, Wahlbrink A, Anish C, Witzenrath M, et al. Development of an Efficacious, Semisynthetic Glycoconjugate Vaccine Candidate against Streptococcus pneumoniae Serotype 1. ACS central science. 2018;4(3):357-61.

304. Lepenies B, Seeberger PH. The promise of glycomics, glycan arrays and carbohydrate-based vaccines. Immunopharmacol Immunotoxicol. 2010;32(2):196-207.

305. Kuttel MM, Jackson GE, Mafata M, Ravenscroft N. Capsular polysaccharide conformations in pneumococcal serotypes 19F and 19A. Carbohydr Res. 2015;406C:27-33.

306. Hlozek J, Kuttel MM, Ravenscroft N. Conformations of Neisseria meningitidis serogroup A and X polysaccharides: The effects of chain length and O-acetylation. Carbohydr Res. 2018;465:44-51.

307. Lakkitjaroen N, Takamatsu D, Okura M, Sato M, Osaki M, Sekizaki T. Capsule loss or death: The position of mutations among capsule genes sways the destiny of Streptococcus suis. FEMS Microbiol Lett. 2014;354(1):46-54.

308. Auger JP, Meekhanon N, Okura M, Osaki M, Gottschalk M, Sekizaki T, et al. Streptococcus suis Serotype 2 Capsule In Vivo. Emerg Infect Dis. 2016;22(10):1793-6.

309. Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. 1991;9:271-96.

310. d'Empaire G, Baer MT, Gibson FC. The K1 Serotype Capsular Polysaccharide of *Porphyromonas gingivalis* Elicits Chemokine Production from Murine Macrophages That Facilitates Cell Migration. Infect Immun. 2006;74(11):6236-43.

311. Wang Q, McLoughlin RM, Cobb BA, Charrel-Dennis M, Zaleski KJ, Golenbock D, et al. A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2. J Exp Med. 2006;203(13):2853-63.

312. Simpson SQ, Singh R, Bice DE. Heat-killed pneumococci and pneumococcal capsular polysaccharides stimulate tumor necrosis factor-alpha production by murine macrophages. Am J Respir Cell Mol Biol. 1994;10(3):284-9.

313. Soell M, Diab M, Haan-Archipoff G, Beretz A, Herbelin C, Poutrel B, et al. Capsular polysaccharide types 5 and 8 of Staphylococcus aureus bind specifically to human epithelial (KB) cells, endothelial cells, and monocytes and induce release of cytokines. Infect Immun. 1995;63(4):1380-6.

314. Zughaier SM. Neisseria meningitidis capsular polysaccharides induce inflammatory responses via TLR2 and TLR4-MD-2. J Leukoc Biol. 2011;89(3):469-80.

315. Sen G, Khan AQ, Chen Q, Snapper CM. In Vivo Humoral Immune Responses to Isolated Pneumococcal Polysaccharides Are Dependent on the Presence of Associated TLR Ligands. J Immunol. 2005;175(5):3084-91.

316. Yu J, Briles DE, Englund JA, Hollingshead SK, Glezen WP, Nahm MH. Immunogenic protein contaminants in pneumococcal vaccines. J Infect Dis. 2003;187(6):1019-23.

317. Mayer S, Raulf M-K, Lepenies B. C-type lectins: their network and roles in pathogen recognition and immunity. Histochem Cell Biol. 2017;147(2):223-37.

318. Varki A, Angata T. Siglecs—the major subfamily of I-type lectins. Glycobiology. 2006;16(1):1R-27R.

319. Thiemann S, Baum LG. Galectins and Immune Responses-Just How Do They Do Those Things They Do? Annu Rev Immunol. 2016;34:243-64.

320. Elliott SD, Clifton-Hadley F, Tai J. Streptococcal infection in young pigs. V. An immunogenic polysaccharide from *Streptococcus suis* type 2 with particular reference to vaccination against streptococcal meningitis in pigs. J Hyg (Lond). 1980;85(2):275-85.

321. del Campo Sepulveda EM, Altman E, Kobisch M, D'Allaire S, Gottschalk M. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect ELISA. Vet Microbiol. 1996;52(1-2):113-25.

322. Calzas C, Taillardet M, Fourati I, Roy D, Gottschalk M, Soudeyns H, et al. Evaluation of the immunomodulatory properties of *Streptococcus suis* and Group B *Streptococcus* capsular polysaccharides on the humoral response. Pathogens. 2017;6(2):16.

323. Wisselink HJ, Stockhofe-Zurwieden N, Hilgers LAT, Smith HE. Assessment of protective efficacy of live and killed vaccines based on a non-encapsulated mutant of *Streptococcus suis* serotype 2. Vet Microbiol. 2002;84(1–2):155-68.

324. Varki A, Gagneux P. Multifarious roles of sialic acids in immunity. Ann N Y Acad Sci. 2012;1253(1):16-36.

325. Snapper CM. Distinct Immunologic Properties of Soluble Versus Particulate Antigens. Front Immunol. 2018;9:598.

326. Colino J, Chattopadhyay G, Sen G, Chen Q, Lees A, Canaday DH, et al. Parameters Underlying Distinct T Cell-Dependent Polysaccharide-Specific IgG Responses to an Intact Gram-Positive Bacterium versus a Soluble Conjugate Vaccine. J Immunol. 2009;183(3):1551-9.

327. Kuttel MM, Cescutti P, Distefano M, Rizzo R. Fluorescence and NMR spectroscopy together with molecular simulations reveal amphiphilic characteristics of a Burkholderia biofilm exopolysaccharide. J Biol Chem. 2017;292(26):11034-42.

328. Ravi Kumar MNV. A review of chitin and chitosan applications. Reactive and Functional Polymers. 2000;46(1):1-27.

329. Newman MJ, Balusubramanian M, Todd CW. Development of adjuvant-active nonionic block copolymers. Adv Drug Deliv Rev. 1998;32(3):199-223.

330. Heath PT. An update on vaccination against Group B *Streptococcus*. Expert Rev Vaccines. 2011;10(5):685-94.

331. Baker CJ, Rench MA, Edwards MS, Carpenter RJ, Hays BM, Kasper DL. Immunization of pregnant women with a polysaccharide vaccine of Group B *Streptococcus*. N Engl J Med. 1988;319(18):1180-5.

332. Kasper DL, Paoletti LC, Wessels MR, Guttormsen HK, Carey VJ, Jennings HJ, et al. Immune response to type III Group B streptococcal polysaccharide-tetanus toxoid conjugate vaccine. J Clin Invest. 1996;98(10):2308-14.

333. Moens L, Jeurissen A, Nierkens S, Boon L, Van Kaer L, Kasran A, et al. Generation of antibody responses to pneumococcal capsular polysaccharides is independent of CD1 expression in mice. Infect Immun. 2009;77(5):1976-80.

334. Schütz K, Hughes R, Parker A, Quinti I, Thon V, Cavaliere M, et al. Kinetics of IgM and IgA antibody response to 23-valent pneumococcal polysaccharide vaccination in healthy subjects. J Clin Immunol. 2013;33(1):288-96.

335. Racine R, Winslow GM. IgM in microbial infections: Taken for granted? Immunol Lett. 2009;125(2):79-85.

336. Pereira CL, Geissner A, Anish C, Seeberger PH. Chemical Synthesis Elucidates the Immunological Importance of a Pyruvate Modification in the Capsular Polysaccharide of Streptococcus pneumoniae Serotype 4. Angew Chem Int Ed Engl. 2015;54(34):10016-9.

337. Weishaupt MW, Matthies S, Hurevich M, Pereira CL, Hahm HS, Seeberger PH. Automated glycan assembly of a S. pneumoniae serotype 3 CPS antigen. Beilstein J Org Chem. 2016;12:1440-6.

338. Seeberger PH, Pereira CL, Khan N, Xiao G, Diago-Navarro E, Reppe K, et al. A Semi-Synthetic Glycoconjugate Vaccine Candidate for Carbapenem-Resistant Klebsiella pneumoniae. Angew Chem Int Ed Engl. 2017;56(45):13973-8.

339. Seeberger PH, Pereira CL, Govindan S. Total synthesis of a Streptococcus pneumoniae serotype 12F CPS repeating unit hexasaccharide. Beilstein J Org Chem. 2017;13:164-73.

340. Plotkin SA. Immunologic correlates of protection induced by vaccination. Pediatr Infect Dis J. 2001;20(1):63-75.

341. Plotkin SA. Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis. 2008;47(3):401-9.

342. Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol. 2010;17(7):1055-65.

343. Sommariva M, de Cesare M, Meini A, Cataldo A, Zaffaroni N, Tagliabue E, et al. High efficacy of CpG-ODN, cetuximab and cisplatin combination for very advanced ovarian xenograft tumors. J Transl Med. 2013;11:25.

344. Chu RS, McCool T, Greenspan NS, Schreiber JR, Harding CV. CpG oligodeoxynucleotides act as adjuvants for pneumococcal polysaccharide-protein conjugate vaccines and enhance antipolysaccharide immunoglobulin G2a (IgG2a) and IgG3 antibodies. Infect Immun. 2000;68(3):1450-6.

345. Sen G, Chen Q, Snapper CM. Immunization of Aged Mice with a Pneumococcal Conjugate Vaccine Combined with an Unmethylated CpG-Containing Oligodeoxynucleotide Restores Defective Immunoglobulin G Antipolysaccharide Responses and Specific CD4+-T-Cell Priming to Young Adult Levels. Infect Immun. 2006;74(4):2177-86.

346. Baker CJ, Paoletti LC, Wessels MR, Guttormsen H-K, Rench MA, Hickman ME, et al. Safety and Immunogenicity of Capsular Polysaccharide—Tetanus Toxoid Conjugate Vaccines for Group B Streptococcal Types Ia and Ib. J Infect Dis. 1999;179(1):142-50.

347. Pawlowski A, Kallenius G, Svenson SB. Preparation of pneumococcal capsular polysaccharide-protein conjugate vaccines utilizing new fragmentation and conjugation technologies. Vaccine. 2000;18(18):1873-85.

348. Watanabe H, Numata K, Ito T, Takagi K, Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. Shock. 2004;22(5):460-6.

349. Chia R, Achilli F, Festing MF, Fisher EM. The origins and uses of mouse outbred stocks. Nat Genet. 2005;37(11):1181-6.

350. Martin RM, Brady JL, Lew AM. The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice. J Immunol Methods. 1998;212(2):187-92.

351. Lecours M-P, Letendre C, Clarke D, Lemire P, Galbas T, Benoit-Biancamano M-O, et al. Immune-responsiveness of CD4+ T cells during *Streptococcus suis* serotype 2 infection. Sci Rep. 2016;6:38061.

352. Shulman M, Wilde CD, Kohler G. A better cell line for making hybridomas secreting specific antibodies. Nature. 1978;276(5685):269-70.

353. Strohl WR. Antibody discovery: sourcing of monoclonal antibody variable domains. Current drug discovery technologies. 2014;11(1):3-19.

354. Pollack M, Koles NL, Preston MJ, Brown BJ, Pier GB. Functional properties of isotype-switched immunoglobulin M (IgM) and IgG monoclonal antibodies to Pseudomonas aeruginosa lipopolysaccharide. Infect Immun. 1995;63(11):4481-8.

355. Paizi M, Zivion D, Spira G. Use of mutagens to increase rate of immunoglobulin isotype switching of hybridoma cells. Hybridoma. 1995;14(1):85-90.

356. Roche AM, Richard AL, Rahkola JT, Janoff EN, Weiser JN. Antibody blocks acquisition of bacterial colonization through agglutination. Mucosal Immunol. 2014;8:176.

357. Dalia Ankur B, Weiser Jeffrey N. Minimization of bacterial size allows for complement evasion and is overcome by the agglutinating effect of antibody. Cell Host Microbe. 2011;10(5):486-96.

358. Chang Q, Zhong Z, Lees A, Pekna M, Pirofski L. Structure-Function Relationships for Human Antibodies to Pneumococcal Capsular Polysaccharide from Transgenic Mice with Human Immunoglobulin Loci. Infect Immun. 2002;70(9):4977-86.

359. Fabrizio K, Manix C, Guimaraeš AJ, Nosanchuk JD, Pirofski L-a. Aggregation of *Streptococcus pneumoniae* by a pneumococcal capsular polysaccharide-specific human monoclonal IgM correlates with antibody efficacy in vivo. Clin Vaccine Immunol. 2010;17(5):713-21.

360. Fabrizio K, Manix C, Tian H, van Rooijen N, Pirofski L-a. The efficacy of pneumococcal capsular polysaccharide-specific antibodies to serotype 3 *Streptococcus pneumoniae* requires macrophages. Vaccine. 2010;28(47):7542-50.

361. Weinstein JR, Quan Y, Hanson JF, Colonna L, Iorga M, Honda S-i, et al. IgM-dependent phagocytosis in microglia is mediated by complement receptor 3, not Fcα/μ receptor. J Immunol. 2015;195(11):5309-17.

VII. ANNEXES

ANNEXES - ARTICLE VIII

Atypical *Streptococcus suis* in man, Argentina

Raquel Callejo [1], M. Prieto [1], F. Salamone [2], Jean-Philippe Auger [3], **Guillaume Goyette-Desjardins** [3], and Marcelo Gottschalk [3]

[1] Instituto Nacional de Enfermedades Infecciosas, ANLIS Dr. Carlos G. Malbrán, Buenos Aires, Argentina;

[2] Hospital San Martín, Paraná, Entre Ríos, Argentina;

[3] Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, Québec, Canada

Publié dans *Emerging Infectious Diseases.* **2014**. 20(3):500-502.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé à la recension de la littérature.

Abstract

Streptococcus suis is an important swine and zoonotic agent. We report for the first time the isolation of a *S. suis* serotype 21 from a patient, who presented peritonitis. Although this serotype has already been recovered from diseased pigs, it is rather usually isolated from clinically healthy pigs. The patient denied any recent occupational or even occasional contact with swine or other animals and had no history of eating raw or undercooked pork. Atypical serotypes of *S. suis* rarely present in humans may be difficult to identify biochemically and may sometimes be under-diagnosed.

Introduction

Streptococcus suis is a major swine pathogen responsible for important economic losses to the swine industry worldwide [1]. It is also an emerging zoonotic agent of meningitis, endocarditis and streptococcal toxic shock-like syndrome, among other infections [2]. Since the recent recognition of the high prevalence of *S. suis* human disease in South East and East Asia, the interest of the scientific community on this pathogen has significantly increased [3]. Among the thirty-five serotypes that have been described based on capsular antigens, the serotype 2 is the most frequently isolated from diseased pigs and humans in most countries [1]. Human cases of *S. suis* infections have been documented in several European and Asian countries as well as in North and South America, Australia and New Zealand [2]. In Western countries, *S. suis* infections in humans have usually been restricted to workers in close contact with pigs or pork by-products. However, in South East and East Asia, this pathogen affects not only that population at higher risk but also the general population, and it represents a significant public health concern [3]. In some Asian countries, the backyard type of production of swine as well as open meat markets are very popular. In humans, it is believed that people can become infected through skin lesions handling infected pork meat [2]. However, it has been recently suggested that the oral route of infection may predominate in countries where dishes are prepared with raw pork meat and/or blood, such as Thailand and Vietnam [4]. On the other hand, in some Latin-American countries, although the backyard type of production of pigs is also common, very few reports on human infection are available [5, 6]. In the present case we report a peritonitis case caused by *S. suis* serotype 21 in a patient in Argentina, South America, without known contact with swine or pork-derived products. It is the first time that this atypical serotype is reported in humans.

Case report

A 62 year old man from Paraná, province of Santa Fe, Argentina, who had a history of tobacco and alcohol abuse was admitted to the hospital as an emergency case with the symptoms of acute abdomen. Ten days previously to his admission to the hospital, the patient developed abdominal distention accompanied by a significant upper abdominal pain. The patient's family reported he had been suffering from gastrointestinal bleeding four days before admittance and he was suspected to be diabetic.

On admission, physical examination, chest and abdominal radiography, abdominal ultrasonography, routine serum laboratory tests (including complete white blood cell count, standard liver and renal function tests), ascitic fluid cell count, ascitic and blood cultures and fresh urine sediment were performed. Physical examination revealed jaundice, hepatosplenomegaly and ascites. On neurologic examination the patient was vigil and disoriented and his vital signs were stable. Temperature was 38,9 ºC, pulse rate 130 beats per minute, and blood pressure 110/70 mm Hg. Laboratory findings were 2,900 leukocytes/μL (70% neutrophils); 94,000 platelets/μL; hemoglobin concentration serum 13,20 g/dL; glucose 195 mg/dL; blood urea nitrogen 42 mg/dL; creatinine 0,96 mg/dL; serum bilirrubin 3,01 mg/dL, ALT 35 U/L; AST 70 U/L; serum albumin 2,66 g/dL, and extension of prothrombin time to 22 sec.

Spontaneous bacterial peritonitis was suspected. Abdominal paracentesis was performed resulting in evacuation of turbid milky fluid. The analysis of ascitic fluid demonstrated a protein level of 16 g/L; 1,340 cells/uL (90% neutrophils); a lactate dehydrogenase level of 221 U/L and amylase level of 34 U/L. Samples of blood and ascetic fluid (10 mL each) were inoculated in aerobic and anaerobic blood culture bottles at the patient's bedside. Gram staining was performed and no organisms were observed.

Empirical treatment with intravenous ceftriaxone (2g/day) was started with the diagnosis of spontaneous bacterial peritonitis associated with liver cirrhosis. After 48 h of incubation, blood and ascitic cultures bottles were positive and an aliquot was plated into sheep blood agar and chocolate agar and incubated at 35 ºC in a 5% CO_2 enriched atmosphere. After 24 h incubation, cultures demonstrated growth of α-hemolytic streptococci. An API Strep (bioMérieux Marcy l'Etoile, France) was inoculated according to the manufacturer´s recommendations and after 48 h incubation gave a profile number of 0240453 and an identification of *Streptococcus pneumoniae* with 58,7% probability, *Streptococcus suis* with 20,7% probability and an "unacceptable identification" confidence level.

Species identification was performed by sequence analysis of 16S rRNA gene as described [7]. Serotyping was performed by co-agglutination test with all reference antisera as

previoulsy described [8]. The isolate was confirmed by polyvalent and monovalent reagents to be a serotype 21.

Antimicrobial drug susceptibility testing, performed according to guidelines of the Clinical and Laboratory Standards Institute, indicated susceptibility to penicillin, ceftriaxone, chloramphenicol, tetracycline, erythromycin and vancomycin. Diagnostic paracentesis was repeated every 2 days. Infection was considered resolved when all signs of infection had disappeared, polymorphonuclear cell count in ascitic fluid had decreased to a level less than 250 cells/uL, and ascitic fluid cultures were negative for bacterial growth. Antibiotic therapy was maintained for 48 hours after resolution of infection. The patient denied any recent occupational or even occasional contact with swine or other animals and had no history of eating raw or undercooked pork.

Discussion

S. suis is considered an emerging zoonotic agent. Although meningitis, endocarditis and, more recently, toxic shock-like syndrome are commonly described in humans, other types of infections have also been reported [2]. More precisely, spontaneous peritonitis as that described in the present report have previously been reported, mainly in Vietnam and Thailand [9-12]. One case was caused by a serotype 5, one by serotype 16 and serotype 2 was involved in the two other cases. In the present case report, and for the first time, a serotype 21 was isolated from a case of human peritonitis. The reference strain of this serotype had originally been isolated from the tonsils of a clinically healthy pig [13]. In fact, it has been shown that this serotype is predominant in tonsils of clinically healthy pigs [13]. However, sixteen strains have also been recovered from ill pigs between 2008 and 2011 in Canada [14]. This indicates that this serotype is potentially virulent.

The only two previous reports of *S. suis* isolated from humans in Latin America are, as the present case, from Argentina [5, 6]. Additional strains of *S. suis* serotype 2 have been isolated from patients in this country (unpublished data). Since the swine production in Argentina is relatively small compared to other countries (such as Brazil and Mexico, for

example), the significant isolation rate of this pathogen in this Latin-American country is probably the consequence of a good surveillance system and good knowledge of the pathogen by local diagnostic laboratories. To note that half of serotypes other than serotype 2 (and especially serotypes 9 to 22) are usually not identified as *S. suis* by rapid multitest identification system [15], such as the API 20 Strep, as it was the case of the present report. Direct PCR detection of *S. suis* serotype 2 (the most common serotype recovered from humans) [16] would have also misidentified this case.

Interesting, the association between human *S. suis* infection and occupational exposure has been largely reported in Europe and in the few cases of North America [3]. In Vietnam, the proportion of patients reported to have occupational exposures was lower than reported in European patients but it remained an important independent risk factor [4]. In the present case report, the patient could not remember any contact with swine or pork-derived products. A similar case was also described in Italy [17]. A patient with *S. suis* infection may be unaware or have no memory of previous exposure to animals. Latent infection with a reactivation many years later has also been described [18]. *S. suis* may become an opportunistic pathogen in persons who are under stress or who have immunodeficiency, and it has been increasingly isolated from mammalian species other than pigs, from birds, and from the environment. The patient in this case has a history of alcohol consumption, which has been described as a predisposal factor [4].

As a conclusion, we described the first case of *S. suis* serotype 21 infection in humans. Diagnostic laboratories should be aware that different serotypes of *S. suis* may be involved in human disease and that their identification requires different and complementally approaches, and absence of awareness recent known exposure to pigs or pork-derived products is possible.

Acknowledgements

This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) grant #154280 to MG.

References

1. Gottschalk M. Streptococcocis. In: Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ, editors. Diseases of Swine. 10th ed. Ames, Iowa, USA: Blackwell Publishing; 2012. p. 841-855.

2. Wertheim HF, Nghia HD, Taylor W, Schultsz C. *Streptococcus suis*: an emerging human pathogen. Clin Infect Dis. 2009;48: 617-625.

3. Gottschalk M, Xu J, Calzas C, Segura M. *Streptococcus suis*: a new emerging or an old neglected zoonotic pathogen? Future Microbiol. 2010;5: 371-391.

4. Nghia HD, Tu le TP, Wolbers M, Thai CQ, Hoang NV, Nga TV, et al. Risk factors of *Streptococcus suis* infection in Vietnam. A case-control study. PloS one. 2011;6: e17604.

5. Lopreto C, Lopardo HA, Bardi MC, Gottschalk M. [Primary *Streptococcus suis* meningitis: first case in humans described in Latin America.]. Enfermedades infecciosas y microbiologia clinica. 2005;23: 110.

6. Nagel A, Manias V, Busquets N, Sniadowsky S, Anzardi J, Mendez Ede L. [*Streptococcus suis* meningitis in an immunocompetent patient]. Rev Argent Microbiol. 2008;40: 158-160.

7. Brousseau R, Hill JE, Prefontaine G, Goh SH, Harel J, Hemmingsen SM. *Streptococcus suis* serotypes characterized by analysis of chaperonin 60 gene sequences. Appl Environ Microbiol. 2001;67: 4828-4833.

8. Gottschalk M, Higgins R, Boudreau M. Use of polyvalent coagglutination reagents for serotyping of *Streptococcus suis*. J Clin Microbiol. 1993;31: 2192-2194.

9. Kerdsin A, Dejsirilert S, Sawanpanyalert P, Boonnark A, Noithachang W, Sriyakum D, et al. Sepsis and spontaneous bacterial peritonitis in Thailand. Lancet. 2011;378: 960.

10. Teekakirikul P, Wiwanitkit V. *Streptococcus suis* infection: overview of case reports in Thailand. The Southeast Asian journal of tropical medicine and public health. 2003;34 Suppl 2: 178-183.

11. Vilaichone RK, Vilaichone W, Nunthapisud P, Wilde H. *Streptococcus suis* infection in Thailand. Journal of the Medical Association of Thailand = Chotmaihet thangphaet. 2002;85 Suppl 1: S109-117.

12. Nghia HD, Hoa NT, Linh le D, Campbell J, Diep TS, Chau NV, et al. Human case of *Streptococcus suis* serotype 16 infection. Emerg Infect Dis. 2008;14: 155-157.

13. Gottschalk M, Higgins R, Jacques M, Beaudoin M, Henrichsen J. Characterization of six new capsular types (23 through 28) of *Streptococcus suis*. J Clin Microbiol. 1991;29: 2590-2594.

14. Gottschalk M, Lacouture S, Bonifait L, Roy D, Fittipaldi N, Grenier D. Characterization of *Streptococcus suis* isolates recovered between 2008 and 2011 from diseased pigs in Quebec, Canada. Vet Microbiol. 2013;162: 819-825.

15. Gottschalk M, Higgins R, Jacques M, Beaudoin M, Henrichsen J. Isolation and characterization of *Streptococcus suis* capsular types 9-22. J Vet Diagn Invest. 1991;3: 60-65.

16. Nga TV, Nghia HD, Tu le TP, Diep TS, Mai NT, Chau TT, et al. Real-time PCR for detection of *Streptococcus suis* serotype 2 in cerebrospinal fluid of human patients with meningitis. Diagnostic microbiology and infectious disease. 2011;70: 461-467.

17. Manzin A, Palmieri C, Serra C, Saddi B, Princivalli MS, Loi G, et al. *Streptococcus suis* meningitis without history of animal contact, Italy. Emerg Infect Dis. 2008;14: 1946-1948.

18. Francois B, Gissot V, Ploy MC, Vignon P. Recurrent septic shock due to *Streptococcus suis*. J Clin Microbiol. 1998;36: 2395.

ANNEXES - ARTICLE IX

Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide

---

Marie-Rose Van Calsteren [1], Fleur Gagnon [1], Cynthia Calzas [2], **Guillaume Goyette-Desjardins** [2], Masatoshi Okura [3], Daisuke Takamatsu [3], Marcelo Gottschalk [2], and Mariela Segura [2]

[1] Food Research and Development Centre, Agriculture and Agri-Food Canada, Saint-Hyacinthe, QC J2S 8E3, Canada.

[2] Groupe de recherche sur les maladies infectieuses du porc (GREMIP) and Centre de recherche en infectiologie porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, Saint-Hyacinthe, QC J2S 2M2, Canada.

[3] Bacterial and Parasitic Disease Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan.

Publié dans *Biochemistry and Cell Biology*. **2013**. 91(2):49-58.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé partiellement aux expériences de purification, contrôles de qualité, analyses par lectines et analyses chimiques (30%).

Abstract

The capsular polysaccharide (CPS) of *Streptococcus suis* serotype 14 was purified, chemically modified, and characterized. Sugar and absolute configuration analyses gave the following CPS composition: D-Gal, 3; D-Glc, 1; D-GlcNAc, 1; D-Neu5Ac, 1. The *Sambucus nigra* lectin, which recognizes the Neu5Ac(α2–6)Gal/GalNAc sequence, showed binding to the native CPS. Sialic acid was found to be terminal, and the CPS was quantitatively desialylated by mild acid hydrolysis. It was also submitted to periodate oxidation followed by borohydride reduction and Smith degradation. Sugar and methylation analyses, ^{1}H and ^{13}C nuclear magnetic resonance, and mass spectrometry of the native CPS or of its specifically modified products allowed to determine the repeating unit sequence: [6)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)]Gal(β1–3)Gal(β1–4)Glc(β1–]$_n$. *S. suis* serotype 14 CPS has an identical sialic acid-containing side chain as serotype 2 CPS, but differs by the absence of rhamnose in its composition. The same side chain is also present in group B *Streptococcus* type Ia CPS, except that in the latter sialic acid is 2,3- rather than 2,6-linked to the following galactose. A correlation between the *S. suis* CPS sequence and genes of the serotype 14 *cps* locus encoding putative glycosyltransferases and polymerase responsible for the biosynthesis of the repeating unit is proposed.

Introduction

Streptococcus suis is a major swine pathogen responsible for important economic losses to the swine industry worldwide and an emerging zoonotic agent of meningitis and streptococcal toxic shock-like syndrome (Gottschalk et al. 2010). Since the recent recognition of the high prevalence of *S. suis* human disease in Asia, the interest of the scientific community in this pathogen has significantly increased (Gottschalk et al. 2010). In western countries, *S. suis* infections in humans have been mainly restricted to workers in close contact with pigs or swine by-products (Gottschalk et al. 2007). However, in Southeast and East Asia, this bacterium affects not only that population at risk but also the general population, and it represents a significant public health concern (Gottschalk et al. 2010). In the very last years, as a direct consequence of these intensified research efforts, large amounts of data on putative virulence factors have appeared in the literature (Fittipaldi et al. 2012). Among them, the capsular polysaccharide (CPS) is considered as the most critical for bacterial virulence (Baums and Valentin-Weigand 2009; Fittipaldi et al. 2012). Of the 35 serotypes described, most studies have been done on *S. suis* serotype 2, which is classically associated with swine and human disease (Gottschalk et al. 2010). Indeed, only serotype 2 CPS has been structurally characterized (Van Calsteren et al. 2010).

In addition to serotype 2, *S. suis* serotype 14 has been described as being an important swine pathogen and an emerging zoonotic agent. Human cases of meningitis and severe sepsis with shock and multiple organ failure have been described in western countries, including Canada (Gottschalk et al. 1989; Haleis et al. 2009; Watkins et al. 2001), and many strains are isolated each year from diseased pigs (M. Gottschalk, unpublished observations). More recently, many human cases caused by a single serotype 14 clone have been described in Thailand (Kerdsin et al. 2009). Our knowledge on the pathogenesis of the disease induced by *S. suis* serotype 14 or on the virulence factors involved is scarce. The only information available is the presence of sialic acid in its CPS (Charland et al. 1995; Smith et al. 2000). Sialic acid has been shown to be an important virulence factor for other meningitis-causing bacteria (Severi et al. 2007). The complete genome sequence of this serotype has recently been published (Hu et al. 2011). We report here for the first time the structure determination of *S.*

suis serotype 14 CPS using chemical, chromatographic, and spectroscopic methods. Lectin binding, physicochemical properties, and biosynthesis were also investigated. The CPS structure was compared with that of other streptococcal antigens.

Materials and methods

Capsule production and extraction, and capsular polysaccharide isolation and purification

Streptococcus suis serotype 14 reference strain DAN 13730 was used in this study. Bacteria were grown in 5 ml of Todd-Hewitt broth (THB) (Oxoid, Thermo Fisher Scientific, Nepean, Ont.) during 16 h at 37 °C. After a dilution of 1/40, bacteria were grown in 150 ml of fresh THB for 16 h, then diluted to 6 L in fresh THB, and grown to an optical density (OD) at 540 nm of 0.8. The cells were pelleted by centrifugation at 10 000*g* for 40 min, suspended by repeated pipetting in a buffer containing 33 mmol/L phosphate and 145 mmol/L NaCl pH 8.0, and chilled. The purified CPS was obtained after extraction, precipitation, and gel filtration as described previously (Van Calsteren et al. 2010). Briefly, the cell suspension was autoclaved at 121 °C for 75 min. The supernatant containing the crude capsule was recovered by centrifugation at 9000*g* for 50 min. Extraction with an equal volume of chloroform eliminated lipids, whereas nucleic acids were removed by precipitation by adding $CaCl_2$ to 0.1 mol/L and ethanol to 25% *v*/*v*, and then centrifuged at 7200*g* for 30 min at room temperature. The concentration of ethanol in the supernatant was increased to 80% *v*/*v* to precipitate the CPS. The suspension was kept overnight at 4 °C and then centrifuged at 9100*g* for 30 min at 4 °C. Pellets were dissolved in 50 mmol/L NH_4HCO_3 (40 mL), dialyzed against the same solution for 48 h with a Spectra/Por 2 membrane (Spectrum Laboratories, Rancho Dominguez, Calif.) MWCO 12–14 kDa, and freeze dried. The CPS was further purified by gel filtration chromatography on a XK26-100 column filled with Sephacryl S-400 (GE Healthcare, Uppsala, Sweden) eluted with 50 mmol/L NH_4HCO_3 at a flow rate of 1.3 ml/min using a Knauer Smartline system equipped with a differential refractometer model RI 2300 and an ultraviolet detector model 2600 (Knauer, Berlin, Germany). UV signals were recorded at 254 and 280 nm. Fractions were collected and assayed for CPS by dot enzyme-linked immunosorbant assay (dot-ELISA) with an anti-*S. suis* serotype 14 rabbit polyclonal antibody (1/1000) as the primary antibody and horseradish peroxidase-conjugated (HRP-conjugated)

goat anti-rabbit Fc secondary antibody (1/3000) (Higgins and Gottschalk 1990). Fractions giving a positive response with antibodies but no absorption at 280 and 254 nm were pooled and freeze dried. The purified residue was dissolved in water, dialyzed as above against deionized water for 48 h at 4 °C, and freeze dried.

Quality controls

Nucleic acids were quantified using an ND-1000 spectrometer (Nanodrop, Wilmington, Del.). The absorbance was measured at 230 and 260 nm. Calculations were done with the Nanodrop software. According to the manufacturer, results are reproducible between 2 and 100 ng/μL.

Protein was quantified by the modified Lowry protein assay kit from Pierce (Rockford, Ill.) on 1 mg/mL CPS samples using a standard curve prepared with diluted albumin standards from 1 to 500 or 1000 μg/mL. The calculated limit of detection ($P \leq 0.05$) was 2–5 μg/mL.

C, H, N, and S elemental analysis was performed on a Fisons Instruments SPA, model EA1108, by the Laboratoire d'analyse élémentaire of the Département de chimie of Université de Montréal.

Mild acid hydrolysis

The CPS (12 mg) was heated in 70 mmol/L HCl (1.2 mL) at 60 °C for 250 min, neutralized with 2 mol/L NH_4OH, dialyzed against deionized water for 48 h at 4 °C with a Spectra/Por membrane MWCO 3500 Da (Spectrum Laboratories), and freeze dried.

Physicochemical characterization

Polysaccharides were characterized by size-exclusion chromatography coupled with multi-angle light scattering (SEC–MALS). The chromatographic separation was performed with two 8 mm × 300 mm Shodex OHpak gel filtration columns connected in series (SB-806 and SB-805), preceded by an SB-807G guard column (Showa Denko, Tokyo, Japan). Elution was done with a Waters 510 pump (Waters, Milford, Mass.) using a 0.1 mol/L $NaNO_3$ mobile phase filtered through a 0.02 μm membrane (Whatman, Maidstone, UK), at a flow rate of

0.4 mL/min. Polysaccharide samples were dissolved in the SEC eluent at a concentration of 0.7 mg/mL for the native CPS and 3 mg/mL for the mild acid-hydrolyzed polysaccharide, kept at 4 °C for a maximum of 3 h to minimize the formation of aggregates, and injected with a 100 µL sample loop. Molar masses and radii of gyration were determined with a Dawn EOS MALS detector (Wyatt, Santa Barbara, Calif.). A differential refractometer model RI 410 (Waters) was used as a concentration detector. A refractive index (RI) increment (*dn*/*dc*) of 0.137 mL/g was calculated for 690 nm using data for xanthan at 436 and 546 nm (Brandrup et al. 2005), and the second virial coefficient (A_2) was taken as zero. Calculations were performed with the ASTRA software version 6.0.0.108 (Wyatt) using 14 detectors from angles 34.8° to 163.3° (detectors 5–18) for the native CPS and 13 detectors from angles 34.8° to 152.5° (detectors 5–17) for the mild acid-hydrolyzed polysaccharide.

Sugar analysis by methanolysis

The monosaccharide composition of polysaccharides was determined by methanolysis (York et al. 1986). To 1 mg of sample, 500 µL of 1 mol/L methanolic HCl (prepared using 465 µL of methanol and 35 µL of acetyl chloride) was added, and the solution was heated at 75 °C for 16 h. The cooled solution was evaporated to dryness with a stream of N_2 at 40 °C, and 500 µL of *tert*-butanol was added and evaporated to dryness. The methyl glycosides were acetylated with 200 µL of pyridine and 200 µL of acetic anhydride at 100 °C for 20 min. The cooled solution was partitioned between deionized water and CH_2Cl_2. The organic layer containing the peracetylated methyl glycosides was analyzed by gas chromatography (GC) either with flame ionization detection (FID) or coupled to mass spectrometry (MS) as described below.

Sugar analysis by hydrolysis

Alternatively, the monosaccharide composition was determined by hydrolysis of 1 mg of sample with 2 mol/L trifluoroacetic acid (TFA) (0.6 mL) at 120 °C for 100 min. After evaporation of the solution with N_2 at 40 °C, the residue was reduced and acetylated by a modification of the method of York et al. (1986): Briefly, 20 mg of $NaBH_4$ in 1 mL of NH_4OH (2 mol/L) was added to the residue, the solution was heated for 60 min at 60 °C, then neutralized with acetic acid, evaporated to dryness with a stream of N_2 at 40 °C, and

evaporated four times by adding 500 µL portions of methanol. The residue was acetylated with 200 µL of pyridine and 200 µL of acetic anhydride at 100 °C for 20 min, partitioned with water (5 mL) and CH_2Cl_2 (2 mL), and the organic layer containing the alditol acetates was analyzed by GC–FID and GC–MS.

Determination of absolute configuration

The absolute configuration was determined according to Leontein et al. (1978). The CPS sample (1 mg) was hydrolyzed with 2 mol/L TFA (0.6 mL) at 120 °C for 100 min. The cooled solution was evaporated to dryness with N_2 at 40 °C and *N*-acetylated by adding pyridine (100 µL) and acetic anhydride (100 µL) to the residue. The solution was stirred for 20 min at room temperature and evaporated to dryness with a stream of N_2 at room temperature. (+)-2-Octanol (100 µL) and TFA (45 µL) were added to the residue. The solution was heated for 17 h at 120 °C, cooled, and evaporated to dryness with a stream of N_2 at 50 °C. Octyl glycosides were acetylated and partitioned as described above. The organic layer was analyzed by GC–MS.

Sugar linkage analysis

Sugar linkage positions were determined on the mild acid-hydrolyzed polysaccharide by analysis of the partially methylated alditol acetates (PMAAs). The methylation procedure was performed by a modified method of Cuicanu and Costello (2003) with NaOH and CH_3I in dimethylsulfoxide (DMSO). The sample (1 mg) was dissolved in 0.7 mL of DMSO. Powdered NaOH (60 mg) was added, and the solution was stirred for 10 min. CH_3I (60 µL) was added, and the solution was stirred for 10 min. Deionized water (5 mL) was added, and the solution was extracted three times with CH_2Cl_2 (2 mL). The combined organic phases were washed four times with water (5 mL). The organic phase was evaporated to dryness with a stream of N_2 at 40 °C. The permethylated saccharides were subjected to sugar analysis by hydrolysis as described above.

Gas chromatography analysis

Organic phases obtained after sugar and linkage analyses were analyzed by GC on an Agilent model 7890 gas chromatograph equipped with a 30 m × 0.32 mm, 0.25 µm HP-5

capillary column (Agilent Technologies, Santa Clara, Calif.). For sugar analysis by methanolysis, the temperature program was: initial oven temperature of 50 °C, maintained for 2 min, ramped at 30 °C/min to 150 °C, ramped at 3 °C/min to 230 °C, maintained for 5 min. The temperatures of the injector and the flame ionization detector were 225 °C and 250 °C, respectively. For sugar analysis by hydrolysis and sugar linkage analyses, the temperature program was: initial oven temperature of 140 °C, maintained for 2.5 min, ramped at 5°C/min to 210 °C, maintained for 28 min. The temperatures of the injector and the flame ionization detector were 250 °C and 275 °C, respectively. Effective carbon responses were used for quantification (Sweet et al. 1975). For absolute configuration determination, the temperature was 140 °C for 2.5 min, ramped at 10 °C/min to 230 °C, which was kept constant for 40 min. The temperatures of the injector and the flame ionization detector were 225 and 250 °C, respectively. GC–MS analyses were done with a Varian CP3800 gas chromatograph and a Saturn 2000 mass spectrometer equipped with a 30 m × 0.25 mm, 0.25 μm VF-5MS capillary column (Varian, Palo Alto, Calif.). The temperature was set for the inlet at 250 °C, for the transfer line at 170 °C, and for the trap at 150 °C. For sugar analysis and sugar linkage analysis, the temperature program was: initial oven temperature of 120 °C, maintained for 1.5 min, ramped at 4 °C/min to 200 °C, maintained for 8 min, then ramped at 10 °C/min to 230 °C. For absolute configuration analysis, the temperature program was: initial oven temperature of 150 °C, maintained for 5 min, ramped at 15 °C/min to 190 °C, maintained for 75 min. MS acquisition was in the electron impact mode at 70 eV.

Enzyme-linked lectin assay to detect the presence of sialic acid

In order to verify the presence or absence of sialic acid in the purified native CPS and the mild acid-hydrolyzed polysaccharide, an enzyme-linked lectin assay (ELLA) test was carried out with the *Sambucus nigra* agglutinin (SNA) lectin (Vector Labs, Burlington, Ont.), which specifically recognizes sialic acid as α-Neu5Ac-2,6-D-Gal*p*/Gal*p*NAc (Shibuya et al. 1987), and with *Limax flavus* agglutinin (LFA) (EY Laboratories, San Mateo, Calif.), which recognizes α-Neu5Ac (Miller et al. 1982). The test was based on a previously described technique (Gornik and Lauc 2007), adapted to the CPS. Skimmed milk was used as positive control. Bovine serum albumin (Hyclone, Thermo Fisher Scientific) was used as negative control for LFA. Group B *Streptococcus* (GBS) type V CPS, which possesses an α-Neu5Ac-

2,3-D-Gal*p* sequence, was purified as described (F. Michon and C. Uitz, Baxter International, United States; patent No. US 2007/0154492 A1) and used as negative control for SNA. Briefly, 200 ng of samples (native CPS, mild acid-hydrolyzed polysaccharide, or controls) were added to wells of an ELISA plate (Nunc-Immuno Polysorp, Canadawide Scientific, Toronto, Ont.). After overnight coating at 4 °C, wells were washed and blocked (to avoid nonspecific binding) by the addition of Carbo-Free solution 1x (Vector Labs). After washings, biotin-conjugated SNA or HRP-conjugated LFA lectins (10 µg/mL) was added for 1 h at room temperature (Vector Labs). For SNA only, 0.5 µg/mL HRP-labeled avidin D was added to the wells for 1 h at room temperature (Vector Labs). The enzyme reactions were developed by addition of 3,3',5,5'-tetramethylbenzidine, stopped by addition of 0.5 mol/L H_2SO_4, and the absorbance was read at 450 nm with an ELISA plate reader.

Nuclear magnetic resonance

Methyl glycoside standards were obtained from Sigma-Aldrich (St. Louis, Mo.). The native CPS and 2-*O*-methyl-α-D-*N*-acetylneuraminic acid were exchanged in phosphate buffer (p^{2}H 8.0) in ^2H_2O (99.9 atom% ^{2}H), freeze dried, and dissolved in ^2H_2O (99.96 atom% ^{2}H) to a final phosphate concentration of 34–80 mmol/L. The mild acid-hydrolyzed polysaccharide and the neutral methyl glycosides were exchanged in ^2H_2O (99.9 atom% ^{2}H), freeze dried, and dissolved in ^2H_2O (99.96 atom% ^{2}H). In all cases, the exchange was repeated if necessary. Nuclear magnetic resonance (NMR) spectra were acquired on polysaccharide samples at concentrations of circa 1%–2%. ^{1}H and ^{13}C chemical shifts δ in ppm were both referenced with internal deuterated 2,2-dimethyl-2-silapentane-5-sulfonate (DSS-d_6) at δ 0 as recommended by Wishart et al. (1995).

A Chemagnetics (Fort Collins, Colo.) CMX Infinity 300 spectrometer was used for 7.05 T experiments with 5 mm Nalorac (Martinez, Calif.) probes: inverse *z*-gradient ^{1}H{^{15}N–^{31}P} or dual ^{13}C/^{1}H. For polysaccharide samples, the temperature was controlled at 50 or 60 °C. Experiments were performed with original pulse programs of the Spinsight software.

NMR spectra were also acquired at 11.75 T on a Bruker Avance 500 spectrometer equipped with a 5 mm triple-resonance TBI probe with ^{1}H, ^{13}C, and ^{109}Ag–^{31}P channels at 60 °C and a 16.45 T Bruker Avance 700 spectrometer with a 5 mm Cryoprobe with ^{1}H and ^{13}C channels at 74 or 77 °C in 3 mm tubes using standard Bruker pulse sequences at the Centre régional de résonance magnétique nucléaire of the Département de chimie of Université de Montréal. Conventional ^{1}H spectra were acquired with a 30° pulse. ^{1}H-decoupled ^{13}C experiments were performed using a 30° pulse or with composite-pulse decoupling using a spin-echo with a carbon 180° adiabatic pulse. One-dimensional (1D) distortionless enhancement by polarization transfer (DEPT) spectra were recorded with adiabatic pulses with a reading pulse of 135° and the free-precession period optimized for 160 or 165 Hz one-bond coupling constant. The gradient-enhanced two-dimensional (ge-2D) correlation spectroscopy (COSY) experiment was performed in magnitude mode. The phase-sensitive ge-2D total correlation spectroscopy (TOCSY) with Malcom Levitt's sequence (MLEV) using echo–antiecho or the phase-sensitive 2D TOCSY using MLEV were acquired with an effective spin lock time of 100 or 80 ms, respectively. The phase-sensitive ge-2D rotating-frame nuclear Overhauser effect spectroscopy (ROESY) without TOCSY (T-ROESY) using echo–antiecho was acquired with a mixing time of 150 ms. The magnitude-mode ge-2D heteronuclear multiple-quantum coherence (HMQC) experiment was recorded with a delay optimized for 145 Hz one-bond ^{13}C–^{1}H coupling constant. The phase-sensitive ge-2D heteronuclear single-quantum coherence (HSQC) using *z*-filter and selection before t_1 was optimized for 160 or 165 Hz. The phase-sensitive ge-2D HSQC–TOCSY with MLEV using echo–antiecho was performed with a delay optimized for a 160 Hz coupling constant and a mixing time of 35 or 60 ms. Magnitude-mode ge-2D heteronuclear multiple-bond coherence (HMBC) with or without using low-pass *J*-filter was run without carbon decoupling with one-bond and long-range delays optimized for 160 and 8 Hz, respectively. Spectra were processed off-line with SpinWorks 3.1 (Copyright © 2009, Kirk Marat, University of Manitoba). Zhu-Bax forward–backward linear prediction (Zhu and Bax 1992) was systematically applied to 2D processing in the f_1 dimension.

Periodate oxidation, Smith degradation, and electrospray ionization mass spectrometry

To a solution of 11 mg of polysaccharide dissolved in 6.5 mL of 0.2 mol/L acetate buffer pH 3.7 at 4 °C was added 68 mg of sodium periodate (0.05 mol/L). The solution was allowed to react in the dark at 4 °C for 7 d. Ethylene glycol (1.3 mL) was added, and the solution was kept for 3 h at room temperature. The solution was dialyzed with a Spectra/Por 1 MWCO 6–8 kDa (Spectrum Laboratories) against deionized water for 48 h and freeze dried. The residue was reduced with 0.5 mol/L $NaBH_4$ in 2 mol/L NH_4OH (1.5 mL) at 60 °C for 4 h. The solution was treated with IRA-120H ion-exchange resin (Sigma-Aldrich), filtered, and freeze dried. The residue was desalted using an Econo-Pac 10-DG desalting column (Bio-Rad, Hercules, Calif.) and freeze dried. A portion of the residue (1 mg) was kept to perform sugar analysis by hydrolysis.

The rest of the material was submitted to Smith degradation as follows. The oxidized–reduced residue was subjected to mild acid hydrolysis in 0.5 mol/L TFA (2 mL) at 80 °C for 1 h. The solution was cooled and freeze dried. The residue was reduced for 60 min with 0.5 mol/L $NaBH_4$ in 2 mol/L NH_4OH (1 mL) as after the oxidation. The residue was dialyzed against deionized water for 24 h at room temperature using a Spectra/Por Biotech cellulose ester membrane MWCO 100 Da (Spectrum Laboratories) and freeze dried. The resulting oligosaccharide was analyzed with nano-electrospray ionization tandem mass spectrometry (nano-ESI–MS/MS) carried out in negative- and positive-ion modes on a tandem mass spectrometer (LC-Q-TOF–MS/MS system–Waters Q-Tof SYNAPT MS) equipped with a nanoACUITY UPLC system (Waters). Masslynx software v. 4.1 (Waters) was used for analysis. Nano-ESI voltages were 3.0 and 2.8 kV in positive and negative modes, respectively. Trap collision energies were 6 and 40 V for full-scan MS and MS/MS, respectively. Samples were prepared as in Dell et al. (1992). They were dissolved in 5% acetic acid in deionized water for the positive mode and in 5% ethylenediamine in deionized water for the negative mode. Samples were then diluted with 50:50 deionized water–acetonitrile containing 0.1% formic acid and infused at 0.4 µL/min. Single scans were acquired by scanning between m/z 50 or 100 and 1000.

Results

Capsule extraction and capsular polysaccharide purification

From 6 L of fermentation broth in different experiments, 17–32 mg of CPS was obtained after purification by gel filtration.

Quality controls

Nucleic acid analysis confirmed the absence of significant contamination giving a range of 0.4–0.8% DNA/RNA. Similarly, protein levels were low (0.5–1.8% by weight) in the purified CPS. Elemental analysis of the CPS revealed the presence of carbon, hydrogen, and nitrogen.

Mild acid hydrolysis and physicochemical characterization

Chemical modification of the native CPS was performed by mild acid hydrolysis without complete depolymerization of the polysaccharide. This was demonstrated by physicochemical characterization by SEC–MALS. The native CPS scattered light more intensely than the mild acid-hydrolyzed polysaccharide (see supplementary data Fig. S1A and B),[1] as the mass and the size of the polysaccharide were slightly reduced by mild acid hydrolysis (Table 1).

Chemical analyses

Sugar composition of the CPS, determined after methanolysis and acetylation, gave the ratio 2.3:1:0.9:1.0 for galactose–glucose–glucosamine–neuraminic acid, whereas the ratio was 1:2.9:1.1 for glucose–galactose–glucosamine as determined by hydrolysis followed by reduction and acetylation.

The absolute configurations were D for galactose, glucose, and glucosamine. The possibility of enzymatic hydrolysis with a specific sialidase (Charland et al. 1995) has already confirmed the D configuration for sialic acid.

Linkage positions were confirmed from GC retention times and MS fragmentation patterns of PMAAs (Carpita and Shea 1989). For the mild acid-hydrolyzed polysaccharide, methylation analysis gave the ratio 1.1:1:0.5:0.6:1.0 for the derivatives 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylgalactitol, and 2-acetamido-1,4,5-tetra-*O*-acetyl-2-deoxy-3,6-di-*O*-methylglucitol, indicative of the linkage types terminal Gal, 4-linked Glc, 3-linked Gal, 3,6-linked Gal, and 4-linked GlcN, respectively.

Lectin binding

The SNA lectin, which recognizes the α-Neu5Ac-2,6-D-Gal*p*/Gal*p*NAc sequence (Shibuya et al. 1987), showed strong binding to the native *S. suis* serotype 14 CPS (Fig. 1), indicating that the sialic acid residue is linked at position 6 of galactose. No significant SNA binding to GBS type V CPS was observed (Fig. 1), confirming the specificity of the lectin with the α-Neu5Ac-2,6-D-Gal*p* sequence. The mild acid-hydrolyzed polysaccharide showed a significant, albeit not complete, reduction in SNA binding (Fig. 1). This residual binding might indicate incomplete hydrolysis of sialic acid, but could also be due to SNA interactions with exposed galactose residues in the mild acid-hydrolyzed polysaccharide preparation (Shibuya et al. 1987). To confirm this hypothesis, we performed an ELLA test with the LFA lectin, which recognizes α-Neu5Ac independently of the linkage sequence and which has no binding affinities for galactose (Miller et al. 1982). The LFA lectin showed strong binding to the native CPS but nonsignificant binding to the mild acid-hydrolyzed polysaccharide (Fig. 1), confirming the absence of sialic acid in the latter preparation.

Nuclear magnetic resonance

The ^{1}H NMR spectra of the CPS before and after mild acid hydrolysis are shown in Figure 2. Lines were slightly narrower for the mild acid-hydrolyzed polysaccharide (Fig. 2B), even at lower temperature, probably due to its lower molar mass. Reporter resonance signals were readily identified on both spectra: five anomeric protons in the range δ 4.8–4.4 and acetyl methyl protons of *N*-acetylglucosamine near δ 2.0. Residues were labelled A–E in order of increasing chemical shift of their anomeric protons. Signals characteristic of sialic acid, that is,

acetyl methyl protons near δ 2.0 and methylene protons in position 3 at δ 2.68 and 1.68 (Fig. 2A), disappeared completely after hydrolysis (Fig. 2B), the chemical shift of the lower frequency signal H-3a being characteristic of α-Neu5Ac-(2→6)-β-Gal/α-GalNAc subunits (Machytka et al. 1994). Apparently, signals for all other residues were still present after the acid treatment. The anomeric protons of residues A and E shifted the most towards lower and higher frequencies, respectively; in addition, protons B1 and C1, which were in perfect superposition on the spectrum of the native CPS (Fig. 2A), became resolved after hydrolysis (Fig. 2B). Because hydrolysis led to spectral simplification with better discrimination of signals and slight decrease in line widths and since the only difference between the spectra of the two polysaccharides was the loss of the sialic acid signals, the structure was determined using the mild acid-hydrolyzed desialylated polysaccharide. From the coupling constants ($J_{H\text{-}1–H\text{-}2}$ = 7.6–7.9 Hz) displayed by the anomeric signals (Fig. 2B, Table 2), all five residues have the β anomeric configuration.

The ^{13}C NMR spectrum (see supplementary data Fig. S2A)[1] of the desialylated polysaccharide displays reporter resonance peaks: acetyl carbonyl carbon of *N*-acetylglucosamine at circa δ 177.5, four signals integrating for five anomeric carbons between δ 107 and 105, amino carbon (C-2) of *N*-acetylglucosamine at circa δ 58.1, and acetyl methyl carbon of *N*-acetylglucosamine at circa δ 25.0. The DEPT-135 spectrum (see supplementary data Fig. S2B)[1] shows that only four out of five CH_2 signals were in an unshifted position (δ 64–62) characteristic of hexoses in the pyranose ring form unsubstituted at position 6, whereas the fifth methylene signal was shifted to higher frequencies (δ 71.62), confirming a linkage at position 6 for one sugar residue.

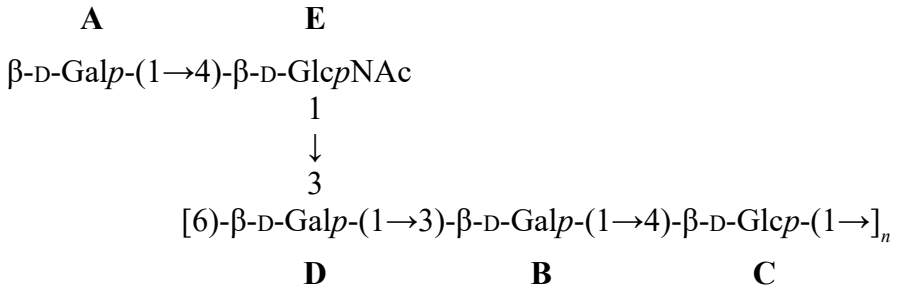
On the COSY spectrum (see supplementary data Fig. S3),[1] starting from the anomeric protons, it was possible to initiate resonance assignments in each spin system in order to identify residues and determine their anomeric configuration. Spins systems could be followed up to H-2 for residues B and E, H-3 for residue C, and H-4 for residues A and D, the latter two being assigned to galactose based on the narrow width of their H-4. The width of the H-1–H-2 cross-peaks confirmed the β anomeric configuration for all residues. Additional cross-peaks can be observed on the COSY spectrum (see supplementary data Fig. S3),[1] very likely

correlations involving H-5 or H-6 for some of the residues, but it was not possible to specifically assign them to individual sugar residues at this stage. Spin systems were further extended with the TOCSY spectrum (Fig. 3). For certain residues, C and E for example, it was possible to follow the spin system from position 1 to position 5 because coupling constants were large and magnetization propagated easily, indicating a β *gluco* configuration. In other cases, as for residues A, B, and D, propagation from the anomeric protons stopped at position 4, which is typical for galactose. Some of the remaining protons could be assigned from the ROESY spectrum (see supplementary data Fig. S4),[1] which clearly displays correlations between H-1 and H-5 for each residue, the two axial protons being in close proximity in sugars with the β anomeric configuration. In the end, a few ambiguities remained as to the assignment of protons in position 6 due to the exact superposition of protons C5 and E5 and to insufficient information (superimposed or near-diagonal cross-peaks) on the COSY spectrum.

From known proton chemical shifts (Table 2), HMQC (not shown) and HSQC (Fig. 4) spectra were used to assign corresponding carbon chemical shifts. The ^{13}C chemical shift of E2 at lower frequency (δ 58.08) indicates that residue E corresponds to *N*-acetylglucosamine, leaving residue C as glucose. At this point, except for a few superimposed signals, carbons 1–5 were assigned for all residues. The HSQC–TOCSY spectrum (see supplementary data Fig. S5)[1] allowed the distinction between resonances at positions 5 and 6 of Glc and GlcNAc (residues C and E), based on correlations at their C-5 chemical shifts. For galactose residues, protons in position 5 previously found on the ROESY spectrum showed long-range correlations to carbons in position 6. However, the small difference in chemical shift for carbons A6 and B6 required another strategy for their differentiation. Interestingly, signals for A1–A4 on the ^{13}C and DEPT spectra had higher intensity than corresponding signals for other residues, and consequently peaks at δ 78.02 and 63.65 could be assigned to A5 and A6, respectively, based on this similar characteristic (Fig. 4 trace). Higher signal intensities are observed for narrower lines, an indication of increased local mobility, which suggests that residue A is located in the side chain of the polysaccharide. On the opposite, resolved carbon signals for residue D had lower than average intensity, implying more restricted motion, suggesting that this residue is located at the branching point.

Careful examination of the carbon chemical shifts in Table 2 gave indication as to the glycosidation position of the sugar residues. When compared to corresponding methyl glycosides, α glycosidation shifts of 7.9–9.1 ppm are observed for carbons B3, C4, D3, D6, and E4. These results confirm the linkage analysis data for all sugars: terminal Gal (A), 4-linked Glc (C), 3-linked Gal (B), 3,6-linked Gal (D), and 4-linked GlcNAc (E). On the ROESY spectrum (see supplementary data Fig. S4),[1] a few inter-residue correlations could readily be identified: E4/A1, C3/B1 or C4/B1, B2/D1, B3/D1, and D3/E1. However, these through-space correlations do not necessarily correspond to linkage positions. In contrast, on the HMBC spectrum (see supplementary data Fig. S6),[1] correlations to anomeric protons (E4/A1, C4/B1, D6/C1, B3/D1, and D3/E1) and, with more ambiguity due to superposition of signals, from anomeric carbons (A1/E4, B1/C4, C1/D6, D1/B3, and E1/D3) confirmed the true linkage positions for all residues.

To ascertain the spectral assignment and the structure determination, methylation analysis data, homonuclear and heteronuclear coupling constant information, and ^{13}C NMR chemical shifts were input into the program CASPER (Lundborg and Widmalm 2011), which outputs possible structures. The following structure had the second lowest root mean square (RMS) deviation between experimental and calculated carbon chemical shifts and is consistent with the connectivity data obtained from the ROESY and HMBC spectra:



Considering that the anomeric proton signal of residues A and E were those that shifted the most upon desialylation, we postulated that sialic acid was linked to position 6 of the terminal galactose residue (A), giving the following as the structure of the native CPS:

α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc
1
↓
3
[6)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→]$_n$

This hypothesis was confirmed by analyzing the differences in the NMR spectra of the two polysaccharides (Tables 2 and 3). On the ^{13}C and DEPT spectrum of the native CPS, additional reporter resonance signals at circa δ 54.7, 42.8, and 24.8 corresponding, respectively, to C-5, C-3, and acetyl methyl of *N*-acetylneuraminic acid were present. In addition, methylene signals at circa 66.0 and 65.6 were attributed to C-6 of a substituted hexose residue and to C-9 of sialic acid, respectively. The following quaternary carbons were found on the ^{13}C spectrum (not shown): three signals in the carbonyl region including two acetyl groups and one carboxylic acid at position 1 of sialic acid, as well as one signal in the anomeric region (δ 103.07) corresponding to C-2 of sialic acid. Starting from the protons in position 3 of sialic acid, the TOCSY spectrum (see supplementary data Fig. S7)[1] displayed new correlations to protons at positions 4–6. The ^{13}C NMR spectrum of the native CPS was assigned from HSQC (see supplementary data Fig. S8)[1] essentially by comparison with that of the desialylated polysaccharide for residues A–E, whereas carbons of the sialic acid residue F were assigned by comparison with the methyl glycoside of *N*-acetylneuraminic acid and with corresponding signals in the *Streptococcus suis* serotype 2 CPS. On the ^{13}C spectrum, carbons of residues F and D have the highest and lowest intensity (see supplementary data Fig. S8 trace),[1] corresponding to the terminal and branching residues, respectively. Carbons A6, B3, C4, D3, D6, and E4 displayed α glycosidation shifts of 2.3–10.1 ppm when compared to the corresponding methyl glycosides. The only ^{13}C chemical shifts to move appreciably (> 1 standard deviation in either direction) upon desialylation were those of carbons A5 (+1.60 ppm), A6 (−2.35 ppm), and E4 (−1.54 ppm), the former two indicating the glycosidation position of the sialic acid on galactose (A6) and the latter presumably being due to different conformations about the A–E glycosidic linkage in the side chain of the two polysaccharides.

Periodate oxidation, Smith degradation, and electrospray ionization mass spectrometry

To ascertain the structure, especially to confirm the sequence E-D-B-C, the CPS was submitted to periodate oxidation followed by borohydride reduction (see supplementary data Scheme S1).[1] Sugar analysis as alditol acetates of the resulting modified polysaccharide gave as a composition glycerol, erythritol, glucose (trace only), galactose, and glucosamine, as expected (ethylene glycol not detected with the temperature program used). The following compound is expected after Smith degradation (mild acid hydrolysis and reduction) (see supplementary data Scheme S1)[1] of the oxidized–reduced polysaccharide:

β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→2)-D-erythritol

ESI–MS analysis of the reaction product gave molecular-ion peaks at *m*/*z* 648.3 and 672.2 in negative- and positive-ion modes, corresponding to the hydrogen-abstracted and sodium-cationized oligosaccharide, respectively. MS/MS of the *m*/*z* 648.3 ion (see supplementary data Fig. S9A)[1] gave peaks at *m*/*z* 445.2, 283.1, and 202.1, corresponding to Y_2, Y_1, and B_1 fragments, respectively, according to the nomenclature of Domon and Costello (1988). In addition, ions corresponding to two fragmentations are also observed: *m*/*z* 179.1 and 161.1. Similarly, the *m*/*z* 672.2 ion (see supplementary data Fig. S9B)[1] fragmented to give peaks at *m*/*z* 550.2, 469.2, 406.1, 388.1, 307.1, 244.1, and 226.1, corresponding to B_3, Y_2, C_2, B_2, Y_1, C_1, and B_1 fragments (see supplementary data Fig. S9C),[1] respectively, and the following ions arose from double fragmentation: *m*/*z* 347.1, 203.1, and 185.0.

Discussion

This report is, to our knowledge, the first description of the CPS structure of *Streptococcus suis* serotype 14.

The reduction in mass upon mild acid hydrolysis of serotype 14 CPS was not as drastic as with serotype 2 CPS (Van Calsteren et al. 2010), which suggests stronger linkages in the polysaccharide structure. This can be explained by the absence in the backbone of serotype 14 CPS of the sugar residue rhamnose, a monosaccharide which is more acid labile than the hexoses glucose and galactose (Hough et al. 1972).

Comparison with other capsular polysaccharide structures

The CPS produced by *S. suis* serotype 14 is unique but shares some common structural elements with other pathogenic streptococcal antigens. *S. suis* serotype 14 CPS differs from that of serotype 2 by the absence of rhamnose in its composition. The sequence Neu5Ac-Gal-GlcNAc-Gal present in *S. suis* serotype 14 CPS is also found in *S. suis* serotype 2 (Van Calsteren et al. 2010) and GBS types Ia, Ib, II, III, and IV CPSs (Cieslewicz et al. 2005) (see supplementary data Fig. S10).[1] Similarly to *S. suis* serotype 2 CPS but contrary to CPSs from GBS types, sialic acid is 2,6- rather than 2,3-linked to the following galactose. The linkages connecting the next three sugars, namely Gal(β1–4)GlcNAc(β1–3)Gal(β1–4), are identical to those in GBS types Ia, II, and III, but one linkage differs in types Ib and IV (see supplementary data Fig. S10).[1] In *S. suis* serotype 14, the side chain is constituted by the sequence Neu5Ac-Gal-GlcNAc, which also forms the side chain in *S. suis* serotype 2 and GBS types Ia, Ib, and IV. In GBS types II and III, however, the sugars in this same sequence are distributed differently between side chain and backbone (see supplementary data Fig. S10).[1] No other pathogenic streptococcal antigen shares common features such as identical composition, backbone, or side chain.

In the CPS of *S. suis* serotype 14, similarly to serotype 2, but differently from GBS type III, sialic acid does not exert conformational control over the backbone residues. When comparing native CPS with their corresponding desialylated polysaccharide, the maximum ^{1}H chemical shift difference for backbone residues is −0.02 ppm for one of the two proton in position 6 of residue D at the branching position, and maximum ^{13}C chemical shift differences of −0.11, +0.09, −0.07, and −0.04 ppm are observed for carbons D6, B3, D5, and D4, respectively. These values are small compared with those found for GBS type III CPS and its corresponding desialylated polysaccharide: +0.06 ppm for Glc H-5; +0.81, +0.26, and +0.21 ppm for GlcNAc C-4, Gal C-3, and Gal C-1, respectively (Brisson et al. 1997). A major difference between the CPS structures of GBS type III and *S. suis* serotypes 2 and 14 is that sialic acid is one residue closer to the backbone in the CPS of GBS type III. Therefore, its influence on the *N*-acetylglucosamine residue is exerted on the backbone in GBS but remains in the side chain in *S. suis*.

Biosynthesis of the capsular polysaccharide

A correlation was tentatively established between the CPS structure determined in this study and seven genes encoding putative glycosyltransferases and a polymerase, which are clustered in the *S. suis* serotype 14 *cps* locus (Hu et al. 2011; Wang et al. 2011) (see supplementary data Table S1 and Fig. 5).[1] In this tentative model, the synthesis of the repeating unit starts with the transfer of the glucose residue to a lipid carrier by the action of the putative glucosyltransferase encoded by *cps14E*, homologous to *cps2E* of *S. suis* serotype 2. The next glycosyltransferase gene (*cps14G*) is homologous to *cpsG* of GBS (also known as *S. agalactiae*) type III, which encodes a β-1,4-galactosyltransferase. From the homology to *cpsI* of GBS type III which encodes an enzyme that catalyzes the formation of a β-D-Glc*p*NAc-(1→3)-β-D-Gal*p* linkage, we propose that the sugar transferred by the gene product of *cps14I* (β-D-Gal*p*) is also β-1,3-linked to galactose. The next two glycosyltransferases, encoded by *cps14J* and *cps14K*, homologous to *cps2J* and *cps2K* of *S. suis* serotype 2, have putative *N*-acetylglucosaminyltransferase and β-1,4-galactosyltransferase activities, respectively. Finally, the last glycosyltransferase gene, *cps14O*, encodes a protein with putative sialyltransferase activity. Remarkably, the order of the glycosyltransferase genes follows that of the sugar sequence (Fig. 5). Finally, the gene product of *cps14H* putatively acts as the CPS polymerase, by homology with the corresponding enzyme in *S. pneumoniae* serotype 14, which also catalyzes the formation of a β-D-Glc*p*-(1→6) linkage, but to β-D-Glc*p*NAc instead of β-D-Gal*p*. It is interesting to note that the absence of rhamnose observed in this study correlates with the absence of a homolog of the *cps2F* gene, which codes for a rhamnosyltransferase, in the *S. suis* serotype 14 *cps* locus (accession number JF273653).

Acknowledgements

We thank Hassan Sabik for the ESI–MS spectra. The LC–MS/MS system was funded by the CRTI technical acquisition program in collaboration with CFIA, AAFC, and PHAC. This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) through grant to MS (#342150-07), by the Fonds québécois de la recherche sur la nature et les technologies (FQRNT)–New Initiative program of the Swine Infectious Disease

Research Centre (CRIP) to MS, MRVC, and MG, and by Grant-in-Aid for Young Scientists (B) from Japan Society for the Promotion of Science (JSPS) to MO. GGD is a recipient of a scholarship from CRIP.

References

Baums, C.G., and Valentin-Weigand, P. 2009. Surface-associated and secreted factors of *Streptococcus suis* in epidemiology, pathogenesis and vaccine development. Anim. Health Res. Rev. **10**(1): 65–83. doi:10.1017/S146625230999003X. PMID:19558750.

Brandrup, J., Immergut, E.H., and Grulke, E.A. (eds). 2005. Polymer handbook. 4th ed. John Wiley & Sons, New York, N.Y.

Brisson, J.-R., Uhrinova, S., Woods, R.J., van der Zwan, M., Jarrell, H.C., Paoletti, L.C., Kasper, D.L., and Jennings, H.J. 1997. NMR and molecular dynamics studies of the conformational epitope of the type III group B *Streptococcus* capsular polysaccharide and derivatives. Biochemistry **36**(11): 3278–3292. doi:10.1021/bi961819l. PMID:9116006.

Carpita, N.C., and Shea, E.M. 1989. Linkage structure of carbohydrates by gas chromatography-mass spectrometry (GC-MS) of partially methylated alditol acetates. *In* Analysis of carbohydrates by GLC and MS. *Edited by* C.J. Bierman, and G.D. McGinnis. CRC Press, Boca Raton, Fla. pp. 157–216.

Charland, N., Kellens, J.T.C., Caya, F., and Gottschalk, M. 1995. Agglutination of *Streptococcus suis* by sialic acid-binding lectins. J. Clin. Microbiol. **33**(8): 2220–2221. PMID:7559986.

Cieslewicz, M.J., Chaffin, D., Glusman, G., Kasper, D., Madan, A., Rodrigues, S., Fahey, J., Wessels, M.R., and Rubens, C.E. 2005. Structural and genetic diversity of group B *Streptococcus* capsular polysaccharides. Infect. Immun. **73**(5): 3096–3103. doi:10.1128/IAI.73.5.3096-3103.2005. PMID:15845517.

Ciucanu, I., and Costello, C.E. 2003. Elimination of oxidative degradation during the per-*O*-methylation of carbohydrates. J. Am. Chem. Soc. **125**(52): 16213–16219. doi:10.1021/ja035660t. PMID:14692762.

Dell, A., Reason, A.J., Khoo, K.-H., Panico, M., McDowell, R.A., and Morris, H.R. 1992. Mass spectrometry of carbohydrate-containing biopolymers. Methods Enzymol. **230**: 108–132. doi:10.1016/0076-6879(94)30010-0. PMID:8139492.

Domon, B., and Costello, C.E. 1988. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. Glycoconjugate J. **5**(4): 397–409. doi:10.1007/BF01049915.

Fittipaldi, N., Segura, M., Grenier, D., and Gottschalk, M. 2012. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent *Streptococcus suis*. Future Microbiol. **7**(2): 259–279. doi:10.2217/FMB.11.149. PMID:22324994.

Gornik, O., and Lauc, G. 2007. Enzyme linked lectin assay (ELLA) for direct analysis of transferin sialylation in serum samples. Clin. Biochem. **40**(9–10): 718–723. doi:10.1016/j.clinbiochem.2007.01.010. PMID:17320850.

Gottschalk, M., Higgins, R., Jacques, M., Mittal, K.R., and Henrichsen, J. 1989. Description of 14 new capsular types of *Streptococcus suis*. J. Clin. Microbiol. **27**(12): 2633–2636. PMID:2480359.

Gottschalk, M., Segura, M., and Xu, J. 2007. *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. Anim. Health Res. Rev. **8**(1): 29–45. doi:10.1017/S1466252307001247. PMID:17692141.

Gottschalk, M., Xu, J., Calzas, C., and Segura, M. 2010. *Streptococcus suis*: a new emerging or an old neglected zoonotic pathogen? Future Microbiol. **5**(3): 371–391. doi:10.2217/FMB.10.2. PMID:20210549.

Haleis, A., Alfa, M., Gottschalk, M., Bernard, K., Ronald, A., and Manickam, K. 2009. Meningitis caused by *Streptococcos suis* serotype 14, North America. Emerg. Infect. Dis. **15**(2): 350–352. doi:10.3201/eid1502.080842. PMID:19193296.

Higgins, R., and Gottschalk, M. 1990. An update on *Streptococcus suis* identification. J. Vet. Diagn. Invest. **2**(3): 249–252. doi:10.1177/104063879000200324. PMID:2094457.

Hough, L., Jones, J.V.S., and Wusteman, P. 1972. On the automated analysis of neutral monosaccharides in glycoproteins and polysaccharides. Carbohydr. Res. **21**(1): 9–17. doi:10.1016/S0008-6215(00)81725-5. PMID:5044142.

Hu, P., Yang, M., Zhang, A., Wu, J., Chen, B., Hua, Y., Yu, J., Xiao, J., and Jin, M. 2011. Complete genome sequence of *Streptococcus suis* serotype 14 strain JS14. J. Bacteriol. **193**(9): 2375–2376. doi:10.1128/JB.00083-11. PMID:21398551.

Kerdsin, A., Oishi, K., Sripakdee, S., Boonkerd, N., Polwichai, P., Nakamura, S., Uchida, R., Sawanpanyalert, P., and Dejsirilert, S. 2009. Clonal dissemination of human isolates of *Streptococcus suis* serotype 14 in Thailand. J. Med. Microbiol. **58**(11): 1508–1513. doi:10.1099/jmm.0.013656-0. PMID:19661209.

Leontein, K., Lindberg, B., and Lönngren, J. 1978. Assignment of absolute configuration of sugars by g.l.c. of their acetylated glycosides formed from chiral alcohols. Carbohydr. Res. **62**(2): 359–362. doi:10.1016/S0008-6215(00)80882-4.

Lundborg, M., and Widmalm, G. 2011. Structural analysis of glycans by NMR chemical shift prediction. Anal. Chem. **83**(5): 1514–1517. doi:10.1021/ac1032534. PMID:21280662.

Machytka, D., Klein, R.A., and Egge, H. 1994. Reporter resonances in the NMR spectra of oligosaccharides containing sialic acid linked to galactopyranose rings. Carbohydr. Res. **254**: 289–294. doi:10.1016/0008-6215(94)84262-0. PMID:8180992.

Miller, R.L., Collawn, J.F., Jr., and Fish, W.W. 1982. Purification and macromolecular properties of a sialic acid-specific lectin from the slug *Limax flavus*. J. Biol. Chem. **257**(13): 7574–7580. PMID:7085639.

Severi, E., Hood, D.W., and Thomas, G.H. 2007. Sialic acid utilization by bacterial pathogens. Microbiology **153**(9): 2817–2822. doi:10.1099/mic.0.2007/009480-0. PMID:17768226.

Shibuya, N., Goldstein, I.J., Broekaert, W.F., Nsimba-Lubaki, M., Peeters, B., and Peumans, W.J. 1987. The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(α2–6)Gal/GalNAc sequence. J. Biol. Chem. **262**(4): 1596–1601. doi:10.1016/S0014-5793(98)00193-8. PMID:3805045.

Smith, H.E., de Vries, R., van't Slot, R., and Smits, M.A. 2000. The *cps* locus of *Streptococcus suis* serotype 2: genetic determinant for the synthesis of sialic acid. Microb. Pathogen. **29**(2): 127–134. doi:10.1006/mpat.2000.0372. PMID:10906268.

Sweet, D.P., Shapiro, R.H., and Albersheim, P. 1975. Quantitative analysis by various g.l.c. response-factor theories for partially methylated and partially ethylated alditol acetates. Carbohydr. Res. **40**(2): 217–225. doi:10.1016/S0008-6215(00)82604-X.

Van Calsteren, M.-R., Gagnon, F., Lacouture, S., Fittipaldi, N., and Gottschalk, M. 2010. Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. Biochem. Cell Biol. **88**(3): 513–525. doi:10.1139/O09-170. PMID:20555393.

Wang, K., Fan, W., Cai, L., Huang, B., and Lu, C. 2011. Genetic analysis of the capsular polysaccharide synthesis locus in 15 *Streptococcus suis* serotypes. FEMS Microbiol. Lett. **324**(2): 117–124. doi:10.1111/j.1574-6968.2011.02394.x. PMID:22092812.

Watkins, E.J., Brooksby, P., Schweiger, M.S., and Enright, S.M. 2001. Septicaemia in a pig-farm worker. Lancet **357**(9249): 38. doi:10.1016/S0140-6736(00)03570-4. PMID:11197360.

Wishart, D.S., Bigam, C.G., Yao, J., Abildgaard, F., Dyson, H.J., Oldfield, E., Markley, J.L., and Sykes, B.D. 1995. ^{1}H, ^{13}C and ^{15}N chemical shift referencing in biomolecular NMR. J. Biomolec. NMR **6**(2): 135–140. doi:10.1007/BF00211777. PMID:8589602.

York, W.S., Darvill, A.G., NcNeil, M., Stevenson, T.T., and Albersheim, P. 1986. Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol. **118**: 3–40. doi:10.1016/0076-6879(86)18062-1.

Zhu, G., and Bax, A. 1992. Improved linear prediction of damped NMR signals using modified "forward–backward" linear prediction. J. Magn. Reson. **100**(2): 202–207. doi:10.1016/0022-2364(92)90379-L.

Tables

Table 1. Size-exclusion chromatography coupled with multi-angle light scattering (SEC–MALS) data for the capsular polysaccharide (CPS) before and after mild acid hydrolysis.

	M_w/M_n	M_w (g/mol)	R_z (nm)
Native CPS	1.025 (0.059%)	2.866×10^5 (0.041%)	18.2 (0.4%)
Mild acid-hydrolyzed polysaccharide	1.022 (0.115%)	1.176×10^5 (0.076%)	11.3 (0.7%)

Note: M_w/M_n, polydispersity; M_w, weight-average molar mass; R_z, z-average radius of gyration. Values in parentheses represent relative standard deviations.

Table 2. Nuclear magnetic resonance (NMR) chemical shifts of the desialylated polysaccharide.

Residue		1[a]	2	3	4	5	6		Me	CO
A	β-D-Gal-(1→	4.48 (7.9)	3.55	3.66	3.93	3.72	3.77			
		105.63	73.73	75.39	71.32	78.02	63.65			
B	→3)-β-D-Gal-(1→	4.50 (7.9)	3.71	3.81	4.18	3.73	3.79	3.77		
		105.31	72.87	84.70	71.06	77.66	63.67			
C	→4)-β-D-Glc-(1→	4.50 (7.7)	3.33	3.64	3.64	3.59	3.98	3.80		
		105.31	75.56	77.18	81.36	77.49	63.06			
D	→3,6)-β-D-Gal-(1→	4.62 (7.7)	3.67	3.73	4.14	3.86	4.02	3.89		
		106.76	72.89	84.48	71.06	75.99	71.62			
E	→4)-β-D-GlcNAc-(1→	4.75 (7.6)	3.77	3.76	3.71	3.59	3.96	3.83	2.03	
		105.18	58.08	74.93	81.48	77.36	62.95		24.96	177.45

Note: Chemical shifts ($^{1}H/^{13}C$) in $^{2}H_2O$ in ppm referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). ^{1}H chemical shifts were obtained at 60 °C from the one-dimensional (1D), correlation spectroscopy (COSY), and total correlation spectroscopy (TOCSY) spectra or at 74 °C from the heteronuclear single-quantum coherence (HSQC) and HSQC–TOCSY spectra. ^{13}C chemical shifts were obtained at 74 °C from the 1D spectrum.

[a]Coupling constants ($J_{H\text{-}1\text{–}H\text{-}2}$) in parentheses.

Table 3. Nuclear magnetic resonance (NMR) chemical shifts of the native capsular polysaccharide (CPS).

Residue		1[a]	2	3	4	5	6	7	8	9	Me	CO
A	→6-β-D-Gal-(1→	4.45 (7.9)	3.54	3.66	3.93	3.80	3.96	3.61				
		105.96	73.59	75.31	71.22	76.42	66.00					
B	→3)-β-D-Gal-(1→	4.50 (7.9)	3.71	3.81	4.18	3.73	3.79	3.77				
		105.32[b]	72.87	84.78	71.06	77.64	63.68					
C	→4)-β-D-Glc-(1→	4.50 (7.9)	3.33	3.64	3.64	3.58	3.98	3.81				
		105.29[b]	75.56	77.19	81.36	77.51	63.05					
D	→3,6)-β-D-Gal-(1→	4.62 (7.6)	3.67	3.74	4.15	3.86	4.03	3.87				
		106.77	72.91	84.48	71.02	75.91	71.52					
E	→4)-β-D-GlcNAc-(1→	4.77 (7.2)	3.78	3.79	3.66	3.60	3.95	3.83			2.05	
		105.08	57.96	74.91	83.02	77.19	63.20				25.07	177.45
F	α-D-Neu5Ac-(2→			2.68 1.68	3.68	3.78	3.71	3.55	3.87	3.86 3.65	2.03	
		175.93	103.07	42.83	70.92	54.74	75.30	71.35	74.48	65.59	24.78	177.64

Note: Chemical shifts ($^{1}H/^{13}C$) in 80 mmol/L phosphate buffer $p^{2}H$ 8.0 in $^{2}H_2O$ at 77 °C in ppm referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). ^{1}H chemical shifts were obtained from the one-dimensional (1D), total correlation spectroscopy (TOCSY), and heteronuclear single-quantum coherence (HSQC) spectra. ^{13}C chemical shifts were obtained from the 1D spectrum.

[a]Coupling constants ($J_{H\text{-}1–H\text{-}2}$) in parentheses.

[b]Tentative assignments.

Figures

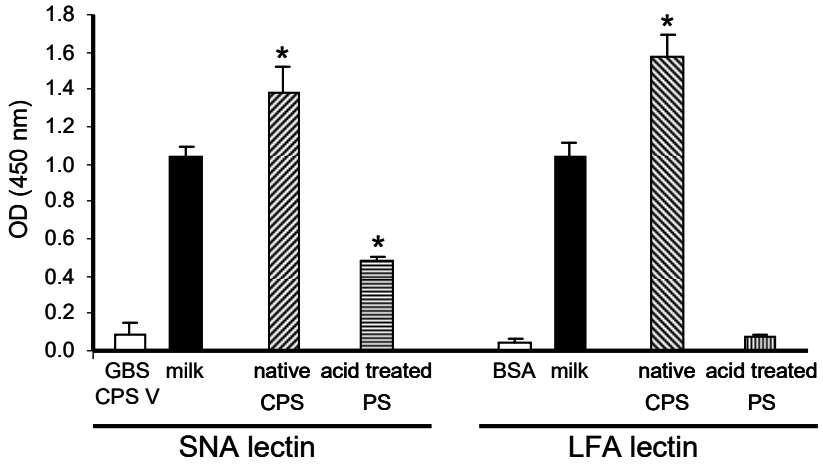


Figure 1. Enzyme-linked lectin assay (ELLA) of *Sambucus nigra* agglutinin (SNA) lectin or *Limax flavus* agglutinin (LFA) binding to purified native capsular polysaccharide (CPS) and mild acid-hydrolyzed polysaccharide (acid treated PS). Skimmed milk was used as positive control (Milk). Bovine serum albumin was used as negative control (BSA) for LFA. Group B *Streptococcus* (GBS) type V purified CPS, which possesses an α-Neu5Ac-2,3-D-Gal*p* sequence, was used as negative control (GBS CPS V) for SNA. Lectin binding was determined by optical density (OD) reading at 450 nm with an enzyme-linked immunosorbant assay (ELISA) plate reader. All data are expressed as mean ± standard error of the mean ($n = 3$). Data were analyzed for significance using Student's unpaired *t*-test. A P value < 0.05 was used as a threshold for significance. *, indicates statistically significant differences compared with the negative control.

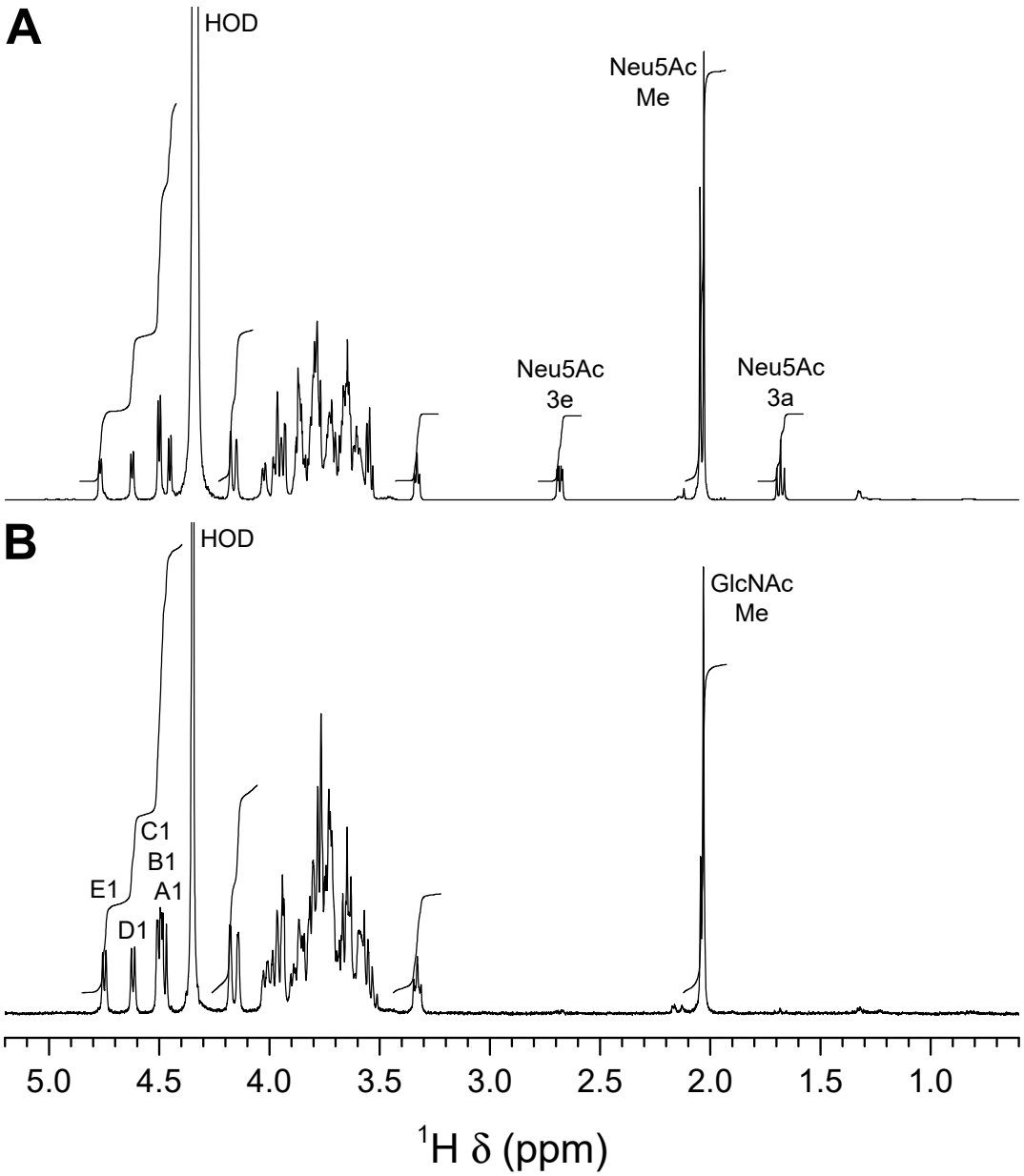


Figure 2. One-dimensional (1D) ^{1}H nuclear magnetic resonance (NMR) spectra.

(A) 700.3 MHz spectrum of the native capsular polysaccharide (CPS) in 80 mmol/L phosphate buffer (p^{2}H 8.0) in $^{2}H_2O$ at 77 °C; 16 K complex data points were acquired with a digital resolution of 0.31 Hz/point and processed by exponential multiplication, zero filling, Fourier transform, phase correction, and third-order polynomial baseline correction. (B) 500.1 MHz spectrum of the mild acid-hydrolyzed polysaccharide in $^{2}H_2O$ at 60 °C; 24 K complex data points were acquired with a digital resolution of 0.25 Hz/point and processed as above.

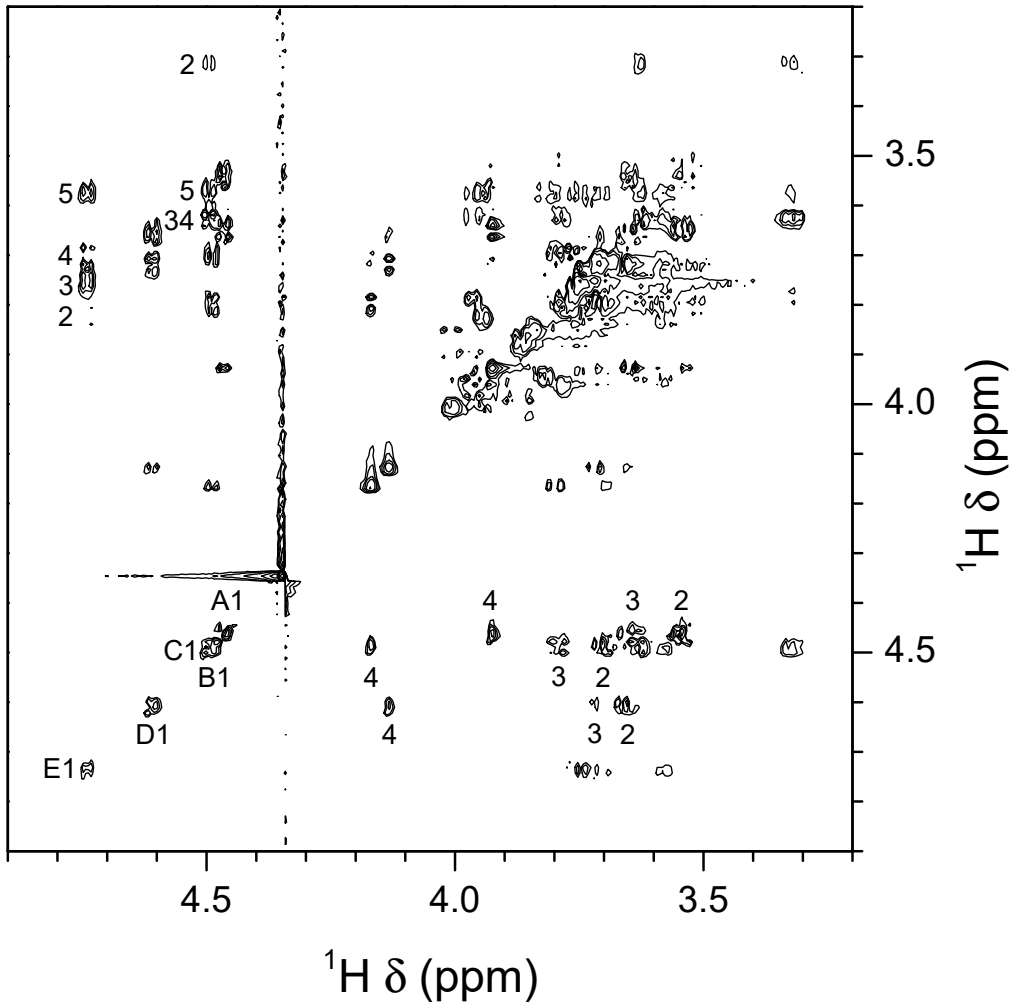


Figure 3. Portion of the 500.1 MHz total correlation spectroscopy (TOCSY) spectrum.

The gradient-enhanced two-dimensional (ge-2D) spectrum of the desialylated polysaccharide was obtained in ^2H_2O at 60 °C. 2 × 256 increments of 1 K complex data points were acquired using echo–antiecho with a mixing time of 100 ms and a digital resolution of 2.7 Hz/point in the t_2 dimension and 11.0 Hz/point in the t_1 dimension. The t_2 dimension was processed by multiplication with a π/2 shifted sinebell window function, Fourier transform, and phase correction, and the t_1 dimension by Zhu-Bax linear prediction to 512 points with 16 coefficients, multiplication with a π/2 shifted sinebell window function, Fourier transform, and phase correction. Only positive contours are shown. Spin systems for residues with the *galacto* and *gluco* configurations are labelled horizontally and vertically, respectively.

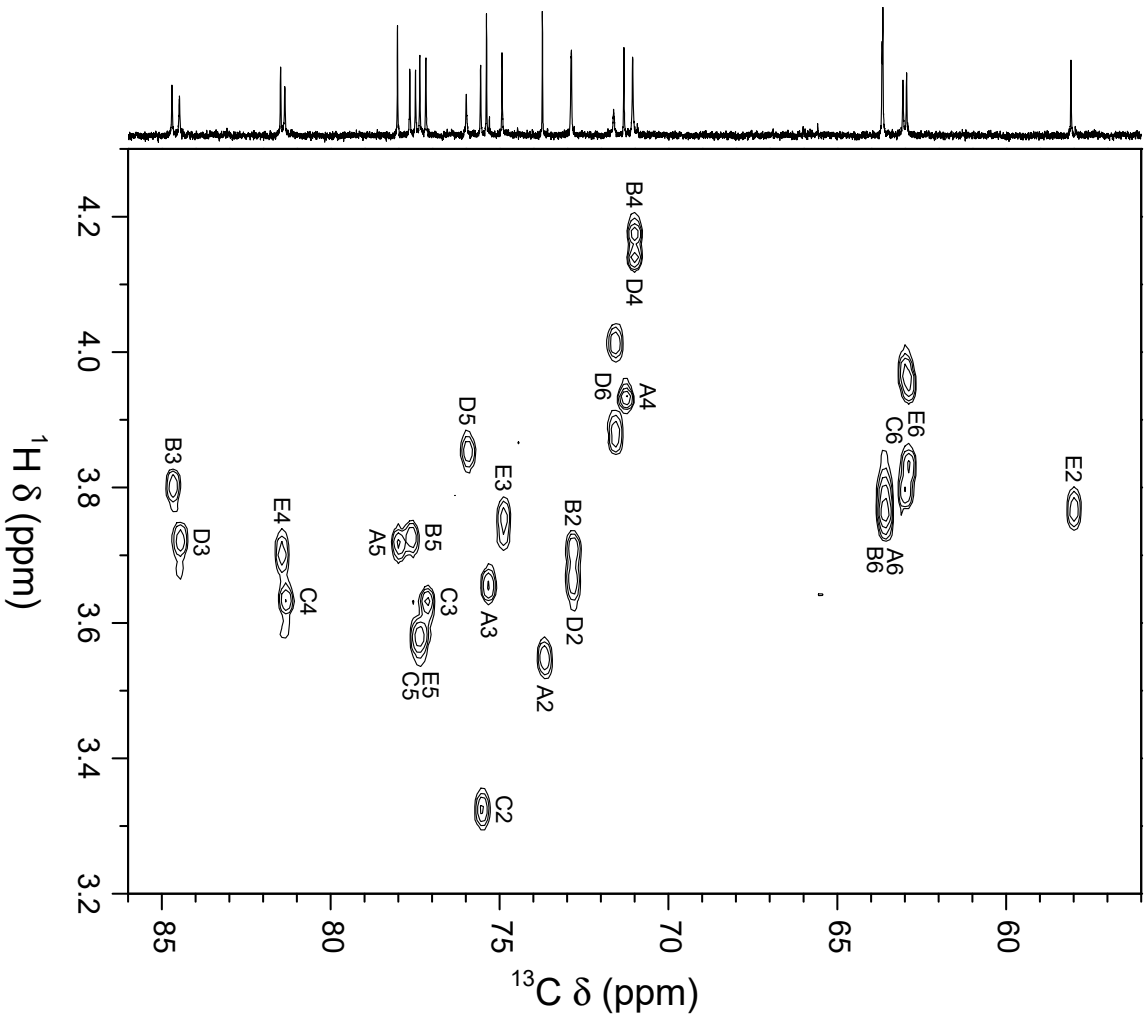


Figure 4. Portion of the 700.3 MHz heteronuclear single-quantum coherence (HSQC) spectrum.

The gradient-enhanced two-dimensional (ge-2D) spectrum of the desialylated polysaccharide was obtained in ^2H_2O at 74 °C. 2 × 180 increments of 768 complex data points were acquired in the States with time-proportional phase incrementation (States-TPPI) mode with a digital resolution of 9.1 Hz/point in the t_2 dimension and 55.6 Hz/point in the t_1 dimension. The t_2 dimension was processed by multiplication with a π/2 shifted sinebell window function, zero filling, Fourier transform, and phase correction, and the t_1 dimension by Zhu-Bax linear prediction to 512 points with 16 coefficients, multiplication with a π/2 shifted sinebell window function, Fourier

transform, and phase correction. Only positive contours are shown. The f_1 trace corresponds to the one-dimensional (1D) spectrum (see supplementary data Fig. S2A).

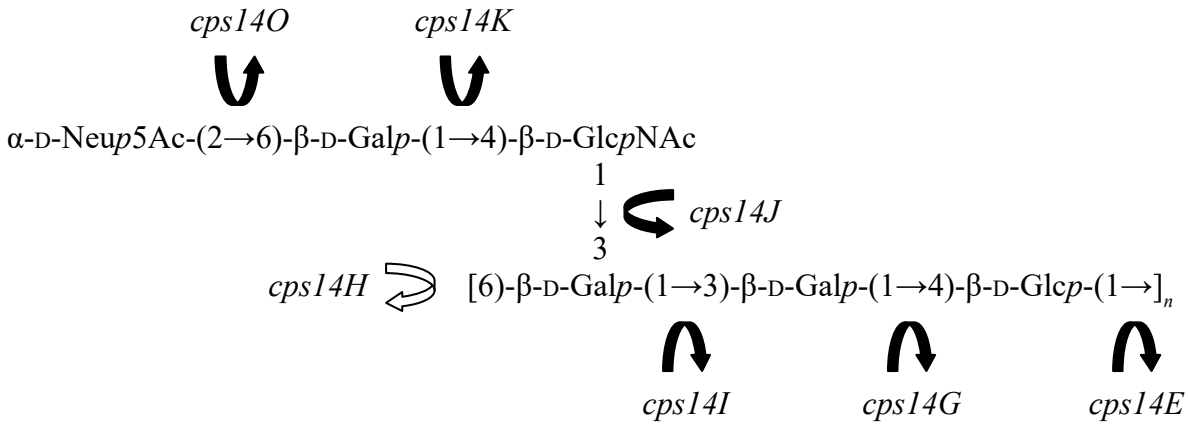


Figure 5. Tentative correlation between structure and genes encoding glycosyltransferases (black arrows) and polymerase (white arrow) responsible for the biosynthesis of *Streptococcus suis* serotype 14 capsular polysaccharide (CPS).

Supporting information

Supplementary material is available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/bcb-2012-0036.

Figure S1. Size-exclusion chromatograms with multi-angle light scattering (MALS) and refractive index (RI) detection.

Figure S2. Regions of the 176.1 MHz one-dimensional 13C nuclear magnetic resonance (NMR) spectra of the desialylated polysaccharide in 2H2O at 74 °C.

Figure S3. Portion of the 500.1 MHz correlation spectroscopy (COSY) spectrum.

Figure S4. Portion of the 500.1 MHz rotating-frame nuclear Overhauser effect spectroscopy without total correlation spectroscopy (T-ROESY) spectrum.

Figure S5. Portion of the 700.3 MHz heteronuclear single-quantum coherence total correlation spectroscopy (HSQC–TOCSY) spectrum.

Figure S6. Portions of the 700.3 MHz heteronuclear multiple-bond coherence (HMBC) spectrum showing correlations to anomeric protons (A) and from anomeric carbons (B).

Figure S7. Portions of the 700.3 MHz total correlation spectroscopy (TOCSY) spectrum.

Figure S8. Portion of the 700.3 MHz heteronuclear single-quantum coherence (HSQC) spectrum.

Scheme S1. Reaction scheme for the preparation of the oligosaccharide.

Figure S9. Mass spectra of the oligosaccharide obtained by Smith degradation of the oxidized–reduced polysaccharide.

Figure S10. Structures of pathogenic streptococci capsular polysaccharides (CPSs) having features in common with that of *Streptococcus suis* serotype 14.

ANNEXES - ARTICLE X

Explaining the Serological Characteristics of *Streptococcus suis* Serotypes 1 and 1/2 from their Capsular Polysaccharide Structure and Biosynthesis

---

Marie-Rose Van Calsteren [1,2], **Guillaume Goyette-Desjardins** [2,3], Fleur Gagnon [1], Masatoshi Okura [4], Daisuke Takamatsu [4,5], René Roy [6], Marcelo Gottschalk [2,3], and Mariela Segura [2,3]

[1] Food Research and Development Centre, Agriculture and Agri-Food Canada, Saint-Hyacinthe, Quebec J2S 8E3, Canada; [2] Swine and Poultry Infectious Disease Research Centre and [3] Research Group on Infectious Diseases of Swine, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, Quebec J2S 2M2, Canada; [4] Bacterial and Parasitic Diseases Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305 0856, Japan; [5] The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Gifu 501 1193, Japan; [6] Department of Chemistry, Université du Québec à Montréal, Montreal, Quebec H3C 3P8, Canada

Publié dans *Journal of Biological Chemistry*. **2016**. 291(16):8387-8398.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé activement aux expériences de purification, contrôles de qualité, analyses chimiques, physicochimiques, sérologiques et avec les lectines (50%), ainsi qu'à l'analyse des résultats et à la rédaction de l'article.

Abstract

The capsular polysaccharide (CPS) is a major virulence factor in many encapsulated pathogens, as it is the case for *Streptococcus suis*, an important swine pathogen and emerging zoonotic agent. Moreover, the CPS is the antigen at the origin of *S. suis* classification into serotypes. Hence, analyses of the CPS structure are an essential step to dissect its role in virulence and the serological relations between important serotypes. Here, the CPSs of serotypes 1 and 1/2 were purified and characterized for the first time. Chemical and spectroscopic data gave the repeating unit sequences: [6)[Neu5Ac(α2–6)GalNAc(β1–4)GlcNAc(β1–3)]Gal(β1–3)Gal(β1–4)Glc(β1–]$_n$.
(serotype 1) and [4)[Neu5Ac(α2–6)GalNAc(β1–4)GlcNAc(β1–3)]Gal(β1–4)[Gal(α1–3)]Rha(β1–4)Glc(β1–]$_n$ (serotype 1/2). The *Sambucus nigra* lectin, which recognizes the Neu5Ac(α2–6)Gal/GalNAc sequence, showed binding to both CPSs. Compared to previously characterized serotypes 14 and 2 CPSs, *N*-acetylgalactosamine replaces galactose as the sugar bearing the sialic acid residue in the side chain. Serological analyses of the cross-reaction of serotype 1/2 with serotypes 1 and 2 and that between serotypes 1 and 14 suggested that the side chain, and more particularly the terminal sialic acid, constitutes one important epitope for serotypes 1/2 and 2. The side chain is also an important serological determinant for serotype 1, yet sialic acid seems to play a limited role. In contrast, the side chain does not seem to be part of a major epitope for serotype 14. These results contribute to the understanding of the relationship between *S. suis* serotypes and provide the basis for improving diagnostic tools.

Introduction

Streptococcus suis is one of the most important pathogens in the porcine industry causing septicemia, meningitis, and many other infections. It is also an emerging zoonotic pathogen. *S. suis* is a normal inhabitant of the upper respiratory tract of pigs. The biochemical identification of isolates recovered from clinically healthy pigs (mainly from tonsil samples) is difficult to achieve due to the presence of other streptococci that are part of the normal oral microflora and that are phenotypically similar to *S. suis*. For this reason, molecular biology techniques have been developed during the last decade to allow the detection and identification of *S. suis* strains at the species level (1). However, serotyping, which should be a part of the routine identification of *S. suis* strains recovered from diseased pigs and humans, is absolutely required to provide further confirmation regarding the pathogen identity. To date, 35 serotypes have been originally described based on capsular polysaccharide (CPS)[2] antigenic diversity, and most of them have been isolated from diseased pigs (2-5). Yet, evidence accumulated throughout the years has demonstrated a high level of genetic diversity in the *S. suis* species, leading to the reclassification of several serotypes (such as serotypes 26, 32, and 34) to different species (1). Globally, the top 10 predominant *S. suis* serotypes isolated from clinical cases in pigs are, in decreasing order, serotypes 2, 9, 3, 1/2, 7, 8, 4, 22, 5, and 1 along with around 15% of the strains being non-typable. However, there is a clear geographical effect on the distribution of serotypes (1). In North America, serotypes 2 and 3 are the most prevalent followed by serotype 1/2 (1). Among virulent serotypes, serotype 2 and, to a lesser extent, serotype 14 are considered serious and high-risk emerging zoonotic agents and their accurate identification is fundamental for diagnostic laboratories.

Proper serological typing must be performed using either a coagglutination test, capillary precipitation test, or with Neufeld's capsular reaction using reference antisera (3, 4). The coagglutination test is preferred by many laboratories, especially in North America (6). Some serotypes cross-react, indicating the presence of common antigenic determinants. This cross-reaction is probably due to similar or closely related structural features of the CPS. To date, important cross-reactions have been described, including serotype 1/2 with serotypes 1 and 2, and between serotypes 1 and 14 (3, 6, 7). The *cps*

loci, in particular the genes encoding putative glycosyltransferases and polymerases, are known for these four serotypes (8). Genetically, the *cps* loci of serotypes 2 and 1/2 are almost identical, and no sequence differences that may contribute to the antigenic divergences were observed between the two serotypes so far (9). Although the *cps* loci of serotypes 1 and 14 are also almost identical, a putative glycosyltransferase gene (*cps1G*) of the serotype 1 reference strain and a sequenced Chinese strain had a frameshift mutation, whereas the corresponding gene (*cps14G*) of the serotype 14 reference strain and a sequenced Chinese strain was intact. These results implied that the mutation in *cps1G* might contribute to the antigenic differences between serotypes 1 and 14 (9); however, no further analyses have been done to investigate this hypothesis. Indeed, a major drawback in recently developed PCR-based typing systems is the fact that the serotypes 2 and 14 cannot be distinguished from serotypes 1/2 and 1, respectively, since both of these serotype pairs do not possess unique *cps* genes (1, 9).

In order to understand the important role of the CPS in serotyping and in the interactions of *S. suis* with the host, knowledge regarding the specific structure that confers capsular properties to each individual serotype is necessary. So far, the CPS repeating unit structure has been determined only for serotypes 2 and 14 (10, 11). Our hypothesis is that the structure of serotype 1 CPS is highly similar to that of serotype 14 CPS but somehow different from that of serotype 2 CPS, whereas serotype 1/2 CPS contains structural elements found mainly in serotype 2 CPS and, to a lesser extent, in serotype 1 CPS. Here, the CPSs of serotypes 1 and 1/2 were purified and characterized for the first time. Serological analyses of the cross-reaction of serotype 1/2 with serotypes 1 and 2 and that between serotypes 1 and 14 suggested that the side chain, constitutes one important antigenic determinant for serotypes 1, 1/2 and 2. In contrast, serotype 14 CPS specifically elicits an antibody response against its backbone. These results contribute to the understanding of the relationship between *S. suis* serotypes and provide the basis for improving diagnostic tools.

Experimental Procedures

Capsular polysaccharide isolation and purification

S. suis serotype 1 strain 1178027, isolated from the meninges of a diseased pig in Canada (unpublished), and serotype 1/2 strain 2651, isolated from a diseased pig in Denmark (2), were used in this study. Bacterial growth, CPS extraction and purification were performed essentially as previously described (10, 11). Gel filtration fractions giving a differential refractometer signal but no UV absorption at 280 and 254 nm were considered to be CPS. This was further confirmed by dot-blotting of all fractions with anti-*S. suis* serotype 1 or 2 serum (6).

Quality controls

Nucleic acids and proteins were quantified essentially as previously described (10, 11).

Mild acid hydrolysis

In order to obtain desialylated polysaccharides for structural and serological studies, mild acid hydrolyses were performed essentially as previously described (10).

Physicochemical characterization

Size-exclusion chromatography coupled with multi-angle light scattering (SEC–MALS) experiments were performed essentially as previously described (10).

Chemical and GC analyses

Sugar analyses by methanolysis and hydrolysis, determination of absolute enantiomeric configuration, glycosidic linkage analysis, and GC or GC−MS analysis were performed essentially as previously described (10).

Enzyme-linked lectin assay to detect the presence of sialic acid

In order to verify the presence or absence of sialic acid in the purified native CPSs and the mild acid-hydrolyzed polysaccharides, an enzyme-linked lectin assay (ELLA) test was carried out with the *Sambucus nigra* agglutinin (SNA) lectin (Vector Labs,

Burlington, ON, Canada), which specifically recognizes sialic acid as α-Neu5Ac-2,6-D-Gal*p*/Gal*p*NAc (12). Skimmed milk was used as positive control. Group B *Streptococcus* (GBS) type V CPS, which possesses an α-Neu5Ac-2,3-D-Gal*p* sequence, was purified as previously described (13) and used as negative control. The assay was performed as previously described (13).

NMR spectroscopy

Native CPSs and the 2-*O*-methyl-α-D-*N*-acetylneuraminic acid standard were exchanged in phosphate buffer (pD 8.0) in D_2O (99.9 atom% D), freeze dried, and dissolved in D_2O (99.96 atom% D) to a final phosphate concentration of 33 mmol/L. The mild acid-hydrolyzed polysaccharide and the neutral methyl glycoside standards were exchanged in D_2O (99.9 atom% D), freeze dried, and dissolved in D_2O (99.96 atom% D). NMR spectra were acquired on polysaccharide samples at concentrations of *ca.* 1%. ^{1}H and ^{13}C chemical shifts δ in ppm were both referenced with internal deuterated 2,2-dimethyl-2-silapentane-5-sulfonate (DSS-d_6) at δ 0 as recommended by Wishart et al. (14).

NMR spectra were acquired at 11.75 T on a Bruker Avance 500 spectrometer equipped with a 5-mm triple-resonance TBI probe with ^{1}H, ^{13}C, and ^{109}Ag–^{31}P channels at 65 °C and a 16.45 T Bruker Avance 700 spectrometer with a 5-mm Cryoprobe with ^{1}H and ^{13}C channels at 42, 56, or 70 °C using standard Bruker pulse sequences at the Regional Centre of NMR of the Department of Chemistry (University of Montreal). Conventional ^{1}H one-dimensional (1D) spectra were acquired with 30° or 90° pulses without or with water presaturation, respectively. The *z*-restored spin-echo was used to acquire ^{1}H-decoupled ^{13}C 1D spectrum of straight baseline. The 1D distortionless enhancement by polarization transfer (DEPT) spectrum was recorded with a reading pulse of 135° and the free-precession period optimized for 145-Hz one-bond coupling constant. The gradient-enhanced two-dimensional (ge-2D) COSY experiment was performed in magnitude mode. The phase-sensitive 2D total correlation spectroscopy (TOCSY) with Malcom Levitt's sequence (MLEV) was acquired with an effective spin lock time of 80 or 100 ms. The phase-sensitive 2D rotating-frame nuclear Overhauser

effect spectroscopy (ROESY) using purge pulses before the relaxation delay, the phase-sensitive 2D ROESY with presaturation, and the phase-sensitive ge-2D ROESY without TOCSY (T-ROESY) using echo–antiecho were acquired with a mixing time of 300 ms. The phase-sensitive ge-2D heteronuclear single-quantum coherence (HSQC) using *z*-filter and selection before t_1 and the phase-sensitive ge-2D HSQC using echo–antiecho and adiabatic pulses for inversion and refocusing and Bloch-Siegert effects were optimized for 145 Hz. The phase-sensitive ge-2D HSQC–TOCSY with MLEV using echo–antiecho was performed with a delay optimized for a 140-Hz coupling constant and a mixing time of 80 or 100 ms. The magnitude-mode ge-2D heteronuclear multiple-bond coherence (HMBC) using low-pass *J*-filter and the phase-sensitive ge-2D HMBC using a three-fold low-pass *J*-filter were run without carbon decoupling with one-bond and long-range delays optimized for 145 and 8 Hz, respectively. Spectra were processed off-line with SpinWorks (Copyright, Kirk Marat, University of Manitoba [http://home.cc.umanitoba.ca/~wolowiec/spinworks/]). Zhu-Bax forward–backward linear prediction (15) with 16 coefficients was systematically applied to 2D processing in the f_1 dimension.

Serological analysis

Serotyping of reference strains by the coagglutination test, which is considered as the "GSGold Standard"' in *S. suis* strain identification and typing, was performed at the International Reference Laboratory for *S. suis* Serotyping (University of Montreal). The reaction, which is done using formalin-fixed whole bacteria cultures with reference monospecific polyclonal rabbit sera against *S. suis* serotype 1, 1/2, 2, or 14, was performed as previously described (6). In addition, dot-blot analyses were performed as previously described by Calzas et al. (13). Ten microliters of purified native CPS or desialylated polysaccharide (each at 1 mg/mL in 50 mmol/L NH_4HCO_3) for serotypes 1, 2, 1/2, and 14 were blotted on a PVDF Western blot membrane (Roche Diagnostics, Basel, Switzerland). The membrane was blocked for 1 h with a solution of TBS containing 2% casein, followed by 2 h of incubation with monospecific polyclonal rabbit sera (6). The membrane was washed, and anti-rabbit HRP-conjugated antibody (Jackson Immunoresearch, West Grove, PA) was added for 1 h. The membrane was washed 3

times with TBS and revealed with a 4-chloro-1-naphthol solution (Sigma-Aldrich). Densitometric analysis of dot-blot scans was performed using ImageJ version 1.49 (16). Raw integration values were normalized using respective homologous signal values with native CPS.

Results

Capsular polysaccharide purification yield and quality controls

From 6 L of fermentation broth in different experiments, CPS yields after purification by gel filtration were 31–44 mg for serotype 1 and 72–75 mg for serotype 1/2. Nucleic acid analysis confirmed the absence of significant contamination giving 0.4% and 0.2% DNA/RNA for serotypes 1 and 1/2, respectively. Similarly, no significant level of protein contamination was detected in the purified CPSs.

Mild acid hydrolysis and physicochemical characterization

Chemical modification of the native CPSs was performed by mild acid hydrolysis without complete depolymerization of the polysaccharide. This was demonstrated by physicochemical characterization by SEC–MALS. The native CPSs scattered light more intensely than the mild acid-hydrolyzed polysaccharides, as the mass and the size of the polysaccharides were reduced by mild acid hydrolysis. For serotype 1, weight average molar masses (M_w) of 7.410×10^5 g/mol and 1.567×10^5 g/mol were determined for the native and hydrolyzed polysaccharides, respectively. For serotype 1/2, M_w of 7.086×10^5 g/mol and 2.249×10^4 g/mol were determined for the native and hydrolyzed polysaccharides, respectively.

Chemical analyses

Sugar composition of the CPSs determined after methanolysis and acetylation gave the ratios 0.0:1.6:1:0.6:0.9:1.1 and 1.1:1.4:1:0.4:1.0:0.5 for Rha:Gal:Glc:GalN:GlcN:Neu for serotypes 1 and 1/2, respectively, whereas the ratios 0.0:1:1.7:0.8:0.9 and 1.0:1:1.7:0.6:0.7 for Rha:Glc:Gal:GlcN:GalN were obtained following hydrolysis, reduction, and acetylation. The data are consistent with repeating

units composed of 1 Glc, 2 Gal, 1 GlcN, 1 GalN, and 1 Neu for serotype 1 CPS and 1 Glc, 2 Gal, 1 Rha, 1 GlcN, 1 GalN, and 1 Neu for serotype 1/2 CPS.

In all cases, the absolute enantiomeric configurations were D for galactose, glucose, glucosamine, and galactosamine, and L for rhamnose. The possibility of enzymatic hydrolysis with a specific sialidase has already confirmed the D configuration for sialic acid for both CPSs (17).

Linkage positions were confirmed from GC retention times and MS fragmentation patterns of partially methylated alditol acetates (18). For the mild acid-hydrolyzed polysaccharide obtained from serotype 1 CPS, methylation analysis gave the ratio 1:0.5:0.5:0.9:1.0 for the derivatives 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylgalactitol, 2-acetamido-1,5-di-*O*-acetyl-2-deoxy-2,3,4,6-tetra-*O*-methylgalactitol and 2-acetamido-1,4,5-tri-*O*-acetyl-2-deoxy-2,3,6-tri-*O*-methylglucitol, indicative of the linkage types 4-linked Glc, 3-linked Gal, 3,6-linked Gal, terminal GalN, and 4-linked GlcN, respectively. Derivatives 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,3,4,5-tetra-*O*-acetyl-6-deoxy-2-*O*-methylmannitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol, 2-acetamido-1,5-di-*O*-acetyl-2-deoxy-2,3,4,6-tetra-*O*-methylgalactitol and 2-acetamido-1,4,5-tri-*O*-acetyl-2-deoxy-2,3,6-tri-*O*-methylglucitol, corresponding to the linkage types terminal Gal, 3,4-linked Rha, 4-linked Glc, 3,4-linked Gal, terminal GalN, and 4-linked GlcN, respectively, were detected for the mild acid-hydrolyzed polysaccharide obtained from serotype 1/2 CPS.

Lectin binding

The SNA lectin, which recognizes the Neu5Ac(α2–6)Gal/GalNAc sequence (12), showed binding to both native CPSs, indicating that the sialic acid residue is linked at position 6 of a galactose or an *N*-acetylgalactosamine residue (Fig. 1).

NMR spectroscopy of serotype 1

The 1D ^{1}H NMR spectrum of serotype 1 CPS (Fig. 2A) displayed five anomeric signals, three acetyl methyl signals, and two methylene signals characteristic of sialic acid at δ 2.68 and 1.68. The chemical shift of the latter axial H-3 is consistent with a 2,6 linkage to a galactopyranose ring (19). Residues were labelled A–E in order of increasing chemical shift of their anomeric protons. From the large coupling constants displayed by their anomeric signals (Fig. 2A, Table 1), these five residues have the β anomeric configuration. In the above reporter resonance regions, differences with serotype 14 CPS (Fig. 2B) were minor: one H-1 signal shifted to higher frequency and one extra CH_3 signal (δ 2.06) from the *N*-acetylgalactosamine residue was present.

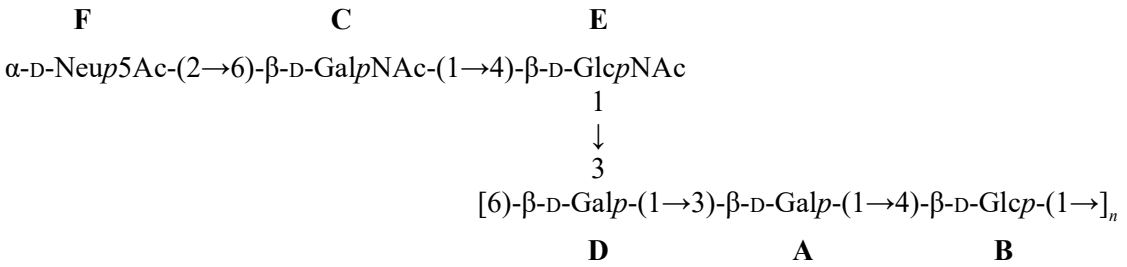
The ^{13}C NMR spectrum (Fig. S1A) displayed reporter resonance peaks: four carbonyl carbons (three acetyl; one carboxyl in position 1 of sialic acid), six anomeric carbons, three amino carbons, one methylene carbon at δ 42.86, and three acetyl methyl carbons. The DEPT-135 spectrum (Fig. S1B) showed that only three out of five CH_2 signals were in an unshifted position, characteristic of hexoses in the pyranose form unsubstituted at position 6, whereas other methylene signals were shifted to higher frequencies (δ 66.06 and 71.47), confirming a linkage at position 6 for two sugar residues. In addition, the methylene signal at δ 65.56 was attributed to C-9 of sialic acid, and one quaternary carbon signal in the anomeric region (δ 103.00) corresponded to C-2 of sialic acid.

On the COSY spectrum (Fig. 3), spins systems could be followed up to H-2 for residues A, D, and E and H-3 for residues B and C. The width of the H-1–H-2 cross-peaks confirmed the β anomeric configuration for residues A–D. Spin systems were further extended with the TOCSY spectrum (Fig. S2). For certain residues, B and E for example, it was possible to follow the spin system from position 1 to positions 5 and 6, because coupling constants were large and magnetization propagated easily, indicating a β *gluco* configuration. In other cases, as for residues A and D, propagation from the anomeric protons stopped at position 4, which is typical for the β *galacto* configuration. Starting from the protons in position 3 of sialic acid, the TOCSY spectrum (Fig. S2)

displayed correlations to protons in positions 4–6. Some of the remaining protons could be assigned from the ROESY spectrum (Fig. S3), which clearly displayed correlations between H-1 and H-5 for residues A–E. With the above additional information from TOCSY and ROESY, further analysis of the COSY spectrum gave nearly complete assignments for most spin systems. In the end, only a few ambiguities remained as to the assignment of protons in position 6 for residues A, B, and D.

The ^{13}C chemical shift of E2 found on the HSQC (Fig. 4) spectrum at lower frequency (δ 57.66) indicates that residue E corresponds to *N*-acetylglucosamine, leaving residue B as glucose. Similarly, the ^{13}C chemical shift of C2 (δ 55.24) indicates that residue C corresponds to *N*-acetylgalactosamine, leaving residues A and D as galactose. Carbons of the sialic acid residue F were assigned by comparison with the methyl glycoside of *N*-acetylneuraminic acid and with corresponding signals in the *S. suis* serotypes 14 and 2 CPSs (10, 11). On the HSQC–TOCSY spectrum (Fig. S4), protons in position 5 previously found on the ROESY spectrum allowed the assignment of protons in position 6, based on long-range correlations at their C-5 chemical shifts, from which corresponding C-6 resonances could be assigned.

When compared to corresponding methyl glycosides, ^{13}C NMR α glycosidation shifts of 2.30–10.63 ppm were observed for carbons A3, B4, C6, D3, D6, and E4. These results confirm the linkage analysis data for all sugars: 3-linked Gal (A), 4-linked Glc (B), 6-linked GalNAc (C) (terminal GalNAc in the desialylated polysaccharide), 3,6-linked Gal (D), 4-linked GlcNAc (E), and terminal Neu5Ac (F). On the ROESY spectrum (Fig. S3), a few inter-residue correlations could readily be identified: B4/A1, D5/B1, D6/B1, E4/C1, E5/C1, A3/D1, D2/E1, and D3/E1. However, these through-space correlations do not necessarily correspond to linkage positions. In contrast, inter-residue correlations found on the HMBC spectrum (Fig. S5), both from anomeric carbons (A1/B4, B1/D6, C1/E4, D1/A3, E1/D3, and F2/C6) and to anomeric protons (B4/A1, D6/B1, E4/C1, A3/D1, and D3/E1), confirmed the true linkage positions for all residues.



Structure of *S. suis* serotype 1 CPS

Finally, the structure was built in the program CASPER [http://www.casper.organ.su.se/casper/] (20), and root mean square deviations between experimental and calculated ^{1}H and ^{13}C chemical shifts were 0.05 and 0.26 ppm, respectively.

NMR spectroscopy of serotype 1/2

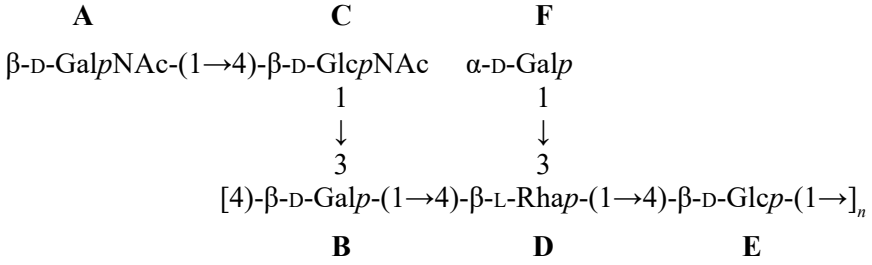
All characteristics of the ^{1}H NMR spectra of serotype 1/2 polysaccharide before and after mild acid hydrolysis (Fig. 5) were identical to those described for the corresponding serotype 2 polysaccharides (11), and similarly, the structure was first determined using the partially hydrolyzed desialylated polysaccharide.

The 1D ^{13}C spectrum (Fig. S6A) displayed reporter resonance peaks: two acetyl carbonyl carbons; six anomeric carbons; two amino carbons (C-2) of *N*-acetylhexosamines; two methyl carbons corresponding to acetyl of *N*-acetylhexosamines and one corresponding to position 6 of rhamnose. Identical information regarding substitution on position 6 and pyranose ring form was obtained from the DEPT-135 spectrum (Fig. S6B) as for the serotype 2 desialylated polysaccharide (11).

Analysis of the COSY (Fig. 6) and TOCSY (Fig. S7) spectra gave essentially the same information about anomeric configuration and identity of residues as for the serotype 2 desialylated polysaccharide (11). As for serotype 1 CPS, the ROESY spectrum (Fig. S8) displayed correlations between H-1 and H-5 for all residues. After full assignment of the proton spectrum (Table 2), the only missing chemical shifts at this stage were those of position 6 of residues A and E.

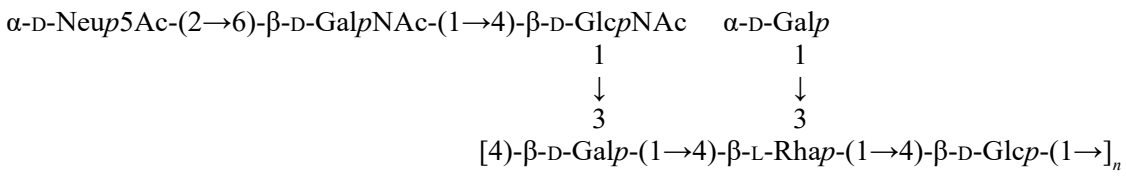
From the ^{13}C chemical shifts of A2 and C2 found on the HSQC (Fig. 7) spectrum at lower frequency (δ 55.33 and 57.99), residues A and C were assigned to *N*-acetylgalactosamine and *N*-acetylglucosamine, respectively, leaving residues B and E as galactose and glucose, respectively. Protons in position 6 of residue E were found on the HSQC–TOCSY spectrum (Fig. S9), and thus the last carbon peak could be assigned to A6 by default.

When compared to corresponding methyl glycosides, α glycosidation shifts of 2.4–9.5 ppm were observed for carbons B3, B4, C4, D3, D4, and E4. These results confirmed the linkage analysis data for all sugars: terminal Gal (F), 3,4-linked Rha (D), 4-linked Glc (E), 3,4-linked Gal (B), terminal GalNAc (A), and 4-linked GlcNAc (C). On the ROESY spectrum (Fig. S8), a few inter-residue correlations could readily be identified: A1/C2–6, B1/D3–4, C1/B3, E1/B4, and F1/D2. These through-space correlations do not necessarily correspond to linkage positions, in part because TOCSY peaks were not suppressed. In contrast, on the HMBC spectrum (Fig. S10), C4, D4, B3, E4, B4, and D3 were confirmed as the true linkage positions for residues A–F, respectively. The following structure is consistent with the connectivity data obtained from the HMBC and ROESY spectra:



Structure of *S. suis* serotype 1/2 desialylated polysaccharide

By analogy with serotype 2 CPS (11), we postulated that sialic acid was linked to position 6 of the terminal *N*-acetylgalactosamine residue (A), giving the following as the structure of the serotype 1/2 native CPS:



This hypothesis was confirmed by analyzing the differences in the NMR spectra of the two polysaccharides (Tables 2 and 3). On the DEPT-135 spectrum of the native CPS (not shown), additional reporter resonance signals at *ca.* δ 54.7, 42.9, and 24.8 corresponding respectively to C-5, C-3, and acetyl methyl of *N*-acetylneuraminic acid were present. In addition, methylene signals at *ca.* δ 66.1 and 65.5 were attributed to C-6 of a substituted hexosamine residue and to C-9 of sialic acid, respectively. The following quaternary carbons were found on the ^{13}C spectrum (not shown): four signals in the carbonyl region belonging to three acetyls and one carboxyl at position 1 of sialic acid, as well as one signal in the anomeric region (δ 102.91) corresponding to C-2 of sialic acid. The 2D TOCSY spectrum (not shown) gave correlation information identical to that of serotype 2 CPS (11). The ^{13}C NMR spectrum of the native CPS was assigned from HSQC (Fig. S11) essentially by comparison with that of the desialylated polysaccharide for residues A–F, whereas carbons of the sialic acid residue G were assigned as for serotype 1 CPS. Carbons A6, B3, C4, D3, D4, and E4 displayed α glycosidation shifts of 2.4–11.0 ppm when compared to the corresponding methyl glycosides; however, carbon B4 was not observed, likely due to restricted motion of this branching residue. As for serotype 2 (11), the only ^{13}C chemical shifts to move appreciably (>1 SD in either direction) upon desialylation were those of carbons A5 (+1.58 ppm), A6 (−2.48 ppm), and C4 (−1.71 ppm). The following inter-residue correlations were unambiguously present on the ROESY spectrum (not shown): A1/C4–5 and F1/D2. Finally, the HMBC (not shown) allowed assignment of the carbonyl carbons.

Serological characteristics

Table 4 presents the cross-reactions obtained between serotypes 1, 1/2, 2, and 14 of *S. suis* using the internationally recognized coagglutination test (performed at the International Reference Laboratory for *S. suis* Serotyping). Serotype 1 strongly reacts with anti-serotype 14 serum, although a serotype 14 strain remains negative/weak with the anti-serotype 1 serum (Table 4). Serotype 1/2 is defined as being positive for both anti-serotype 1 and 2 sera. Although never used as a reagent for serotyping, an anti-serotype 1/2 serum was produced in rabbits and showed positive reactions with serotype 1 and 1/2 strains but resulted in a negative reaction with the serotype 2 reference strain (Table 4).

In order to better understand these serological cross-reactions and the possible structural elements involved, dot-blot experiments were performed using native and desialylated CPSs (Fig. 8). Complete desialylation of the mild-acid hydrolyzed CPSs was confirmed by NMR (data not shown) and by ELLA (Fig. 1) for serotypes 1 and 1/2. Complete desialylation of serotypes 2 and 14 CPSs has been previously and routinely performed (10, 11, 13, 21).

Anti-serotype 1 serum showed a strong reaction with serotype 1 and a weaker response with serotype 14 for both native and desialylated polysaccharides (Fig. 8A and 8E). Yet, native and desialylated polysaccharides of serotypes 1 and 14 were similarly recognized by anti-serotype 14 serum (Fig. 8B and 8F). The weaker recognition of serotype 14 CPS by anti-serotype 1 serum suggests that the *N*-acetylgalactosamine moiety bearing the sialic acid is part of a major immunogenic sequence for serotype 1 CPS. Thus, substitution of the *N*-acetylgalactosamine residue by a galactose in serotype 14 CPS might result in a lower affinity of an anti-serotype 1 serum for serotype 14, whereas the absence of sialic acid does not show a substantial effect (Fig. 9A). On the other hand, this part of the CPS structure does not seem to play a role in the recognition of serotype 1 CPS by anti-serotype 14 serum. In fact, for serotype 14, the major immunogenic part might reside in the polysaccharide backbone sequence (Fig. 9A), which would explain the equal recognition of serotypes 1 and 14 by anti-serotype 14 serum and the non-recognition of serotype 1/2 CPS (Fig. 8B and 8F). For both anti-serotypes 1 and 14 sera, no detection of serotype 2 CPS occurred, illustrating important structural/conformational (and in consequence antigenic) differences between serotypes 1 and 14 dominant epitopes and those of serotype 2. In agreement with these data, anti-serotype 2 serum did not recognize serotypes 1 and 14 CPSs (Fig. 8C and 8G).

Another important cross-reaction is that between serotype 1/2 CPS and anti-serotypes 1 and 2 sera. Dot-blots showed that only native serotype 1/2 CPS was recognized by anti-serotype 1 serum (Fig. 8A and 8E). Indeed, absence of sialic acid in serotype 1/2 CPS abolished this cross-reaction. Similarly, removal of sialic acid also almost completely abolished recognition of serotype 1/2 CPS by its homologous anti-serum (Fig. 8D and 8H). Inversely, native serotype 1 CPS was strongly recognized by anti-serotype 1/2 serum and absence of sialic acid in serotype 1 CPS considerably reduced this cross-reaction (Fig. 8D and 8H). The only common structure

between these two serotypes is the Neu5Ac-(α2,6)-GalNAc-(β1,4)-GlcNAc side chain (Fig. 9B). Yet, compared to serotype 1, presence of sialic acid in serotype 1/2 seems to play a major role in the immunogenic and cross-reactive properties of this serotype.

Similarly, only native serotype 1/2 CPS was significantly recognized by anti-serotype 2 serum (Fig. 8C and 8G). This cross-reaction was, however, weaker than that observed when using anti-serotype 1 serum (Fig. 8A and 8E). In addition, serotype 1/2 anti-serum was unable to recognize either serotype 2 native CPS or desialylated polysaccharide (Fig. 8D and 8H). This result was in agreement with that obtained with the coagglutination test using formalin-fixed whole *S. suis* serotype 2 (strain S735) culture as antigen (Table 4). The substitution of the *N*-acetylgalactosamine residue by a galactose in serotype 2 CPS side chain [Neu5Ac-(α2,6)-Gal-(β1,4)-GlcNAc] might explain the lower affinity of an anti-serotype 2 serum for serotype 1/2 and the absence of recognition of an anti-serotype 1/2 serum for serotype 2 CPS (Fig. 9B).

Finally, recognition by homologous anti-serotype 2 serum showed a stronger reaction with serotype 2 native CPS than with serotype 2 desialylated polysaccharide (Fig. 8C and 8G), indicating that sialic acid is in fact part of one of the serotype 2 immunogenic sequences. Altogether, these data indicate that the side chain and more particularly the terminal sialic acid, constitutes one important immunogenic structure for serotypes 1/2 and 2. The side chain is also an important serological part for serotype 1 structure, yet sialic acid seems to play a limited role. On the other hand, the side chain does not seem to be part of a major immunogenic reaction for serotype 14 CPS (Fig. 9A–B).

Discussion

The CPS is the major virulence factor for several encapsulated pathogens, and it has been proven to be also the case for *S. suis,* at least for serotypes 2 and 14 (22-24). In addition, the CPS is the antigen at the origin of *S. suis* classification into serotypes. Due to the importance of CPS to bacterial pathogenesis, the analysis of the CPS structure is an essential step not only to understand its role in virulence but also to dissect the serological relations between important serotypes.

The presence of a terminal sialic acid at the lateral chain is a common feature of all described serotypes of GBS, a human pathogen known to induce septicemia and meningitis, two pathologies also observed in *S. suis* infection cases. For GBS, it has been proposed that sialic acid plays important modulatory roles in bacterial interactions with host cells and in the regulation of complement activation (25). Similarly to GBS, it has been suggested, based on chemical, genetic, and/or lectin binding analyses that *S. suis* serotypes 1, 1/2, 2, 6, 13, 14, 15, 16, and 27 might also contain sialic acid in their CPSs (8, 9, 17, 26). The presence of this sugar was recently confirmed by CPS structural analyses in *S. suis* serotypes 2 and 14 (10, 11), and in the present study for serotypes 1 and 1/2. Interestingly, sialic acid forms an α-2,6 linkage with the adjacent sugar in *S. suis* versus an α-2,3 linkage in GBS (10, 11, 27, 28). Yet, the role of sialic acid in *S. suis* interactions with host cells or in modulation of the host immune system has been poorly characterized. It has been suggested that sialic acid moiety of the *S. suis* serotype 2 CPS would be, at least in part, responsible for bacterial recognition by macrophages (29). More recently, it has been demonstrated that capsular sialic acid of *S. suis* serotype 2 binds to swine influenza virus and enhances bacterial interactions with virus-infected tracheal epithelial cells (30, 31). Thus, sialic acid might regulate host–pathogen interactions, yet its actual role in *S. suis* virulence remains to be elucidated.

For both serotypes 1 and 1/2 CPSs, ^{1}H and ^{13}C NMR data are consistent with *N*-acetylgalactosamine replacing galactose as the sugar bearing the sialic acid residue, when compared with serotypes 14 and 2 CPSs, respectively. Indeed, ^{13}C chemical shift differences between *N*-acetylgalactosamine and galactose residues in serotypes 1 and 1/2 CPSs compared to serotypes 14 and 2 CPSs are comparable to those between the corresponding methyl glycosides, methyl 2-acetamido-2-deoxy-β-D-galactopyranoside and methyl β-D-galactopyranoside (Table S1). The impact of this modification in the interactions of different *S. suis* serotypes (1 and 1/2 *vs.* 2 and 14) with host cells or the immune system warrants further studies. In addition, higher yields of CPS were obtained for serotypes 1 and 1/2 compared with serotypes 2 and 14 (10, 11). Chain lengths (as represented by the M_w measured by SEC–MALS) for serotypes 1 and 1/2 CPSs are both higher than those previously reported for serotypes 2 and 14 (10, 11), which might account for these differences in yields.

Another observed difference is that serotypes 1 and 1/2 CPSs behave differently upon mild acid hydrolysis. Indeed, the reduction in mass is much more important for serotype 1/2 CPS than for serotype 1 CPS. This behavior was previously reported for serotype 2 CPS when compared to serotype 14 CPS (10). These observations support the hypothesis that the sugar residue rhamnose in the backbone of serotypes 2 and 1/2 CPSs may be responsible for this behavior, as its linkages are more labile than those of the hexoses glucose and galactose (32).

Precise CPS structure analyses allowed for the first time a tentative explanation of the serological characteristics of *S. suis* serotypes 1, 1/2, 2 and 14. The structure of serotype 1 CPS was found to be sufficiently similar to that of serotype 14 CPS to explain its cross-reactivity with anti-serotype 14 serum and *vice-versa*. Nevertheless, recognition of serotype 14 by anti-serotype 1 serum is weaker than homologous recognition, indicating that substitution of the *N*-acetylgalactosamine residue by a galactose in serotype 14 side chain (Fig. 9A, underlined) affects optimal recognition by anti-serotype 1 serum. These results suggest that the main population of antibodies generated by serotype 1 CPS is directed against the side chain (Fig. 9A, in blue). However, terminal sialic acid seems to be dispensable for antibody recognition in serotype 1 and for the cross-reaction with serotype 14. Albeit the side chain seems to harbor the major/dominant immunogenic part of serotype 1 CPS, we cannot rule out production of antibodies against its backbone structure. This second, minor population of antibodies in the anti-serotype 1 serum can also cross-react to a certain extent with the backbone structure of serotype 14 CPS, being identical to that of serotype 1 CPS (Fig. 9A, in red). On the other hand, the backbone part seems to be dominant in serotype 14 CPS as suggested by equal intensity of homologous (serotype 14) and heterologous (serotype 1) recognition by the anti-serotype 14 serum. Our hypothesis that the backbone epitope is dominant in serotype 14 CPS is further supported by the lack of recognition of serotype 1/2 CPS, which side chain is identical to that of serotype 1 CPS but the backbone structure is different (Fig. 9B). Finally, in spite of identical side chains, anti-serotype 14 serum does not react against serotype 2 CPS, further indicating that serotype 14 CPS specifically elicits antibody response against its backbone (Fig. 9).

Another so far unexplained cross-reaction is that of serotype 1/2 CPS with anti-serotypes 2 and 1 sera. Our present study provided for the first time some insight into these cross-reactions.

The side chain of serotype 1/2 CPS seems to be the target of cross-reactive antibodies present in anti-serotype 1 serum. This can be explained by identical structures of these two serotypes CPS side chains (Fig. 9B). However, sialic acid seems to play an important role in serotype 1/2 CPS immunological recognition, as its removal abolishes not only recognition by the homologous serum but also by anti-serotype 1 and anti-serotype 2 sera. The latter further suggests that the identical backbone structure between serotypes 1/2 and 2 CPSs is, surprisingly, not essentially involved in the cross-reaction between these two serotypes. Investigation of anti-serotype 1/2 serum affinities supports previous conclusions. The main population of antibodies generated by serotype 1/2 CPS seems to be directed against its sialic acid-containing side chain, which explains a weaker cross-reaction with desialylated serotype 1 polysaccharide than that with native serotype 1 CPS.

The structural/conformational characteristics of serotype 2 CPS epitopes seem to be complex and remain hypothetical. For example, antibodies against the side chain of serotype 1, which are mainly present in the anti-serotype 1 serum, do not recognize serotype 2 CPS side chain, but they seem to recognize serotype 14 side chain. Being serotypes 2 and 14 CPS side chains identical (Fig. 9), this observation can only be explained by a structural/conformational control of sialic acid in the serotype 2 CPS side chain, which is not observed in serotype 14, or related to structural/conformational control by the backbone (such as steric interactions with the α-Gal side chain). Nevertheless, homologous recognition of serotype 2 CPS by anti-serotype 2 serum is significantly reduced when sialic acid is removed in this serotype CPS (Fig. 8C). Thus, the side-chain seems to dominate the antibody response generated by serotype 2 CPS. As aforementioned, the lack of cross-reaction between anti-serotype 2 serum and desialylated serotype 1/2 polysaccharide further suggests low levels of anti-backbone antibodies generated by serotype 2 CPS. Finally, the enigmatic complete absence of recognition of serotype 2 CPS by serotype 1/2 anti-serum highlights the complexity of the serotype 2 epitopes. It should be highlighted that our data only provided preliminary elucidation of the cross-reactions observed in *S. suis* serotyping and structural/conformational characteristics of major CPS epitopes remain to be precisely determined by using, for example, a panel of monoclonal antibodies.

Based on structural and genetic homologies with serotypes 14 and 2, respectively, putative glycosyltransferase and polymerase genes in the *cps* loci of serotypes 1 and 1/2 were tentatively assigned to the transfer of specific sugars in the repeating units (Table S2, Fig. 10). As aforementioned, the only structural difference observed between serotypes 2 and 1/2 and that between serotypes 1 and 14 are the sugar bearing the sialic acid in the side chains. The sugar is *N*-acetylgalactosamine in serotypes 1/2 and 1, whereas it is galactose in serotypes 2 and 14 (Fig. 9). The enzymes, which were assigned to the transfer of the sugars (Cps1L, Cps1/2K, Cps2K and Cps14K), were classified into the same homology group (HG11) and annotated as a putative galactosyltransferase (9); however, if this tentative assignment is true, Cps1L and Cps1/2K should function as an acetylgalactosaminyltransferase. In order to find sequence differences that may support this hypothesis, we retrieved amino acid sequences of Cps1L, Cps1/2K, Cps2K and Cps14K from available databases and compared them. However, no sequence differences reasonably accounting for the possible change in function from a putative galactosyltransferase to a putative acetylgalactosaminyltransferase could be delineated from those published sequences (Fig. S12). In Figure 10, we assigned *cps1G* (putative galactosyltransferase) to the transfer of galactose to the first glucose in the repeating unit. However, in the previous study (9), this gene was presumed to be a pseudogene due to a frameshift mutation at least in the serotype 1 reference strain and in a Chinese serotype 1 strain. To solve these discrepancies and confirm precise function of each *cps* gene, further studies including construction of gene-manipulated mutants and analysis of the CPS is needed.

In conclusion, we generated for the first time data on CPS composition and structure that will contribute to understand the cross-reactions observed in serotyping. The correct identification of the serotype may have public health impacts, since serotype 14 is the second type of *S. suis* most commonly isolated from human cases after serotype 2 (1).

Acknowledgments

We thank Adrien Brousse, Camille Soudier, and Nancy Guertin for technical assistance. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a grant to MS (#342150). GGD is a recipient of a NSERC fellowship.

References

1. Goyette-Desjardins G, Auger J-P, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent—an update on the worldwide distribution based on serotyping and sequence typing. Emerging Microbes and Infections [Internet]. 2014; 3:[e45 p.].
2. Perch B, Pedersen KB, Henrichsen J. Serology of capsulated streptococci pathogenic for pigs: six new serotypes of *Streptococcus suis*. Journal of Clinical Microbiology. 1983;17(6):993–6.
3. Gottschalk M, Higgins R, Jacques M, Mittal KR, Henrichsen J. Description of 14 new capsular types of *Streptococcus suis*. Journal of Clinical Microbiology. 1989;27(12):2633–6.
4. Gottschalk M, Higgins R, Jacques M, Beaudoin M, Henrichsen J. Characterization of six new capsular types (23 through 28) of *Streptococcus suis*. Journal of Clinical Microbiology. 1991;29(11):2590–4.
5. Higgins R, Gottschalk M, Boudreau M, Lebrun A, Henrichsen J. Description of six new capsular types (29-34) of *Streptococcus suis*. Journal of Veterinary Diagnostic Investigation. 1995;7(3):405–6.
6. Higgins R, Gottschalk M. An update on *Streptococcus suis* identification. Journal of Veterinary Diagnostic Investigation. 1990;2(3):249–52.
7. Perch B, Kjems E, Slot P, Pedersen KB. Biochemical and serological properties of R, S, and RS streptococci. Acta Pathologica et Microbiologica Scandinavica Section B, Microbiology. 1981;89(3):167–71.
8. Wang K, Fan W, Cai L, Huang B, Lu C. Genetic analysis of the capsular polysaccharide synthesis locus in 15 *Streptococcus suis* serotypes. FEMS Microbiol Lett. 2011;324(2):117–24.
9. Okura M, Takamatsu D, Maruyama F, Nozawa T, Nakagawa I, Osaki M, et al. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: potential mechanisms for generation of capsular variation. Applied and Environmental Microbiology. 2013;79(8):2796–806.
10. Van Calsteren M-R, Gagnon F, Calzas C, Goyette-Desjardins G, Okura M, Takamatsu D, et al. Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide. Biochemistry and Cell Biology = Biochimie et Biologie Cellulaire. 2013;91(2):49–58.
11. Van Calsteren M-R, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. Biochemistry and Cell Biology = Biochimie et Biologie Cellulaire. 2010;88(3):513–25.
12. Shibuya N, Goldstein IJ, Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ. The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(α2–6)Gal/GalNAc sequence. The Journal of Biological Chemistry. 1987;262(4):1596–601.
13. Calzas C, Goyette-Desjardins G, Lemire P, Gagnon F, Lachance C, Van Calsteren M-R, et al. Group B *Streptococcus* and *Streptococcus suis* capsular polysaccharides induce chemokine production by dendritic cells via TLR2- and MyD88-dependent and -independent pathways. Infection and Immunity. 2013;81(9):3106–18.
14. Wishart DS, Bigam CG, Yao J, Abildgaard F, Dyson HJ, Oldfield E, et al. ^{1}H, ^{13}C and ^{15}N chemical shift referencing in biomolecular NMR. Journal of Biomolecular NMR. 1995;6(2):135–40.

15. Zhu G, Bax A. Improved linear prediction of damped NMR signals using modified "forward–backward" linear prediction. Journal of Magnetic Resonance. 1992;100(2):202–7.
16. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature methods. 2012;9(7):671-5.
17. Charland N, Kellens JTC, Caya F, Gottschalk M. Agglutination of *Streptococcus suis* by sialic acid-binding lectins. Journal of Clinical Microbiology. 1995;33(8):2220–1.
18. Carpita NC, Shea EM. Linkage structure of carbohydrates by gas chromatography-mass spectrometry (GC-MS) of partially methylated alditol acetates. In: Biermann CJ, McGinnis GD, editors. Analysis of Carbohydrates by GLC and MS. Boca Raton, Fla.: CRC Press; 1989. p. 157–216.
19. Machytka D, Klein RA, Egge H. Reporter resonances in the NMR spectra of oligosaccharides containing sialic acid linked to galactopyranose rings. Carbohydr Res. 1994;254:289–94.
20. Lundborg M, Widmalm G. Structural analysis of glycans by NMR chemical shift prediction. Analytical Chemistry. 2011;83(5):1514–7.
21. Lecours M-P, Fittipaldi N, Takamatsu D, Okura M, Segura M, Goyette-Desjardins G, et al. Sialylation of *Streptococcus suis* serotype 2 is essential for capsule expression but is not responsible for the main capsular epitope. Microbes and Infection. 2012;14(11):941–50.
22. Charland N, Harel J, Kobisch M, Lacasse S, Gottschalk M. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology. 1998;144(2):325–32.
23. Smith HE, Damman M, van der Velde J, Wagenaar F, Wisselink HJ, Stockhofe-Zurwieden N, et al. Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infection and Immunity. 1999;67(4):1750–6.
24. Roy D, Auger J-P, Segura M, Fittipaldi N, Takamatsu D, Okura M, et al. Role of the capsular polysaccharide as a virulence factor for *Streptococcus suis* serotype 14. Canadian Journal of Veterinary Research. 2015;79(2):141–6.
25. Chang YC, Nizet V. The interplay between Siglecs and sialylated pathogens. Glycobiology. 2014;24(9):818–25.
26. Elliott SD, Tai JY. The type-specific polysaccharides of *Streptococcus suis*. J Exp Med. 1978;148(6):1699–704.
27. Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, et al. Structural and genetic diversity of group B *Streptococcus* capsular polysaccharides. Infection and Immunity. 2005;73(5):3096–103.
28. Berti F, Campisi E, Toniolo C, Morelli L, Crotti S, Rosini R, et al. Structure of the type IX Group B *Streptococcus* capsular polysaccharide and its evolutionary relationship with types V and VI. The Journal of Biological Chemistry. 2014;289(34):23437–48.
29. Segura M, Gottschalk M. *Streptococcus suis* interactions with the murine macrophage cell line J774: adhesion and cytotoxicity. Infection and Immunity. 2002;70(8):4312–22.
30. Wang Y, Gagnon CA, Savard C, Music N, Srednik M, Segura M, et al. Capsular sialic acid of *Streptococcus suis* serotype 2 binds to swine influenza virus and enhances bacterial interactions with virus-infected tracheal epithelial cells. Infection and Immunity. 2013;81(12):4498–508.

31. Wu NH, Meng F, Seitz M, Valentin-Weigand P, Herrler G. Sialic acid-dependent interactions between influenza viruses and *Streptococcus suis* affect the infection of porcine tracheal cells. The Journal of general virology. 2015;96(9):2557–68.
32. Hough L, Jones JVS, Wusteman P. On the automated analysis of neutral monosaccharides in glycoproteins and polysaccharides. Carbohydr Res. 1972;21(1):9–17.

Tables

Table 1. NMR chemical shifts of *S. suis* serotype 1 native CPS.

Chemical shifts ($^{1}H/^{13}C$) in 33 mmol/L phosphate buffer pD 8.0 in D_2O in ppm referenced to internal DSS. ^{1}H chemical shifts were obtained at 70 °C from the 1D, at 65 °C from the COSY or TOCSY, or at 70 °C from the HSQC spectra. ^{13}C chemical shifts were obtained at 70 °C from the 1D spectrum.

Residue		1	2	3	4	5	6	7	8	9	Me	CO
A	→3)-β-D-Gal-(1→	4.49 (7.8)[a]	3.71	3.80	4.17	3.73	3.77					
		105.30[b]	72.87[b]	84.81	71.06	77.65	63.68					
B	→4)-β-D-Glc-(1→	4.49 (7.8)	3.33	3.63	3.64	3.58	3.97	3.80				
		105.29[b]	75.54	77.19	81.28	77.51	63.00					
C	→6)-β-D-GalNAc-(1→	4.52 (8.4)	3.93	3.74	3.93	3.79	4.00	3.61			2.06	
		104.77	55.24	73.43	70.28	76.42	66.06				24.97	177.30
D	→3,6)-β-D-Gal-(1→	4.62 (7.5)	3.66	3.73	4.13	3.85	4.02	3.85				
		106.83	72.88[b]	84.49	70.97	75.85	71.47					
E	→4)-β-D-GlcNAc-(1→	4.74 (7.5)	3.78	3.78	3.55	3.54	3.82	3.64			2.05	
		105.14	57.66	75.11	83.54	76.89	63.03				25.08	177.49
F	α-D-Neu5Ac-(2→			2.68 1.68	3.68	3.77	3.71	3.56	3.88	3.87 3.65	2.03	
		175.97	103.00	42.86	70.87	54.74	75.30	71.31	74.46	65.56	24.77	177.66

[a] Coupling constants ($J_{H\text{-}1–H\text{-}2}$) in parentheses. [b] Tentative assignments.

Table 2. NMR chemical shifts of *S. suis* serotype 1/2 desialylated polysaccharide.

Chemical shifts ($^{1}H/^{13}C$) in D_2O in ppm referenced to internal DSS. ^{1}H chemical shifts were obtained at 56 °C from the 1D, COSY, TOCSY, or HSQC spectra. ^{13}C chemical shifts were obtained at 56 °C from the 1D or HSQC spectra.

Residue		1	2	3	4	5	6		Me	CO
A	β-D-GalNAc-(1→	4.53 (8.3)[a]	3.92	3.75	3.94	3.72	3.78		2.06	
		104.47	55.33	73.43	70.40	78.01	63.64		24.96	177.39
B	→3,4)-β-D-Gal-(1→	4.66 (7.3)	3.65	3.77	4.35	3.68	3.78	3.76		
		105.67	73.2[b]	85.13	77.8[b]	76.9[b]	63.32			
C	→4)-β-D-GlcNAc-(1→	4.74 (7.8)	3.76	3.73	3.61	3.52	3.85	3.66	2.03	
		105.55	57.99	75.19	82.16	77.22	63.07		25.10	177.35
D	→3,4)-β-L-Rha-(1→	4.86	4.39	3.93	3.87	3.50	1.35			
		103.1[b]	68.40	77.99	78.6[b]	73.84	19.94			
E	→4)-β-D-Glc-(1→	4.87	3.32	3.66	3.61	3.49	3.93	3.79		
		104.7[b]	76.3[b]	78.4[b]	80.0[b]	77.15	63.81			
F	α-D-Gal-(1→	5.25 (3.4)	3.89	3.97	3.97	4.20	3.73			
		95.80	70.95	72.24[c]	72.14[c]	73.79	63.91			

[a] Coupling constants ($J_{H\text{-}1\text{–}H\text{-}2}$) in parentheses. [b] Obtained from the HSQC spectrum. [c] Tentative assignments.

Table 3. NMR chemical shifts of *S. suis* serotype 1/2 native CPS.

Chemical shifts ($^{1}H/^{13}C$) in 33 mmol/L phosphate buffer pD 8.0 in D_2O in ppm referenced to internal DSS. ^{1}H chemical shifts were obtained at 42 °C from the 1D, COSY, TOCSY, or HSQC spectra. ^{13}C chemical shifts were obtained at 42 °C from the 1D spectrum.

Residue		1	2	3	4	5	6	7	8	9	Me	CO
A	→6)-β-D-GalNAc-(1→	4.50 (8.1)[a]	3.94	3.74	3.93	3.80	4.02	3.56			2.06	
		104.95	55.18	73.40	70.20	76.44	66.12				24.98	177.41
B	→3,4)-β-D-Gal-(1→	4.69	3.66	3.79	4.36	3.70	3.77	3.74				
		105.72	73.10	85.22	—[b]	76.92	63.33					
C	→4)-β-D-GlcNAc-(1→	4.77	3.78	3.78	3.55	3.55	3.82	3.64			2.05	
		105.55	57.73	75.31	83.87	76.92	63.18				26.27	177.41
D	→3,4)-β-L-Rha-(1→	4.86	4.41	3.93	3.87	3.51	1.35					
		103.29	68.28	77.84	78.43	73.81	19.95					
E	→4)-β-D-Glc-(1→	4.91	3.31	3.67	3.62	3.50	3.94	3.81				
		104.60	76.25	78.43	80.12	77.15	63.74					
F	α-D-Gal-(1→	5.26	3.89	3.99	3.98	4.22	3.73					
		95.60	70.94	72.20[c]	72.15[c]	73.81	63.97					
G	α-D-Neu5Ac-(2→			2.67 1.70	3.66	3.79	3.70	3.56	3.90	3.88 3.64	2.03	
		176.13	102.91	42.87	70.88	54.70	75.31	71.23	74.45	65.48	24.79	177.71

[a] Coupling constant ($J_{H\text{-}1\text{–}H\text{-}2}$) in parentheses. [b] Not determined. [c] Tentative assignments.

Table 4. Serological cross-reactions of *Streptococcus suis* when serotyping by the coagglutination method.

Serotype / Strain	PBS[a]	Reference antiserum			
		Type 1	Type 1/2	Type 2	Type 14
Serotype 1 1178027	-	+++	+++	-	+++
Serotype 1/2 2651	-	++	+++	++	-
Serotype 2 S735	-	-	-	+++	-
Serotype 14 DAN13730	-	+/-	-	-	+++

[a] PBS was used as negative control to ensure that formalin-fixed bacteria were not auto-agglutinating.

Figures

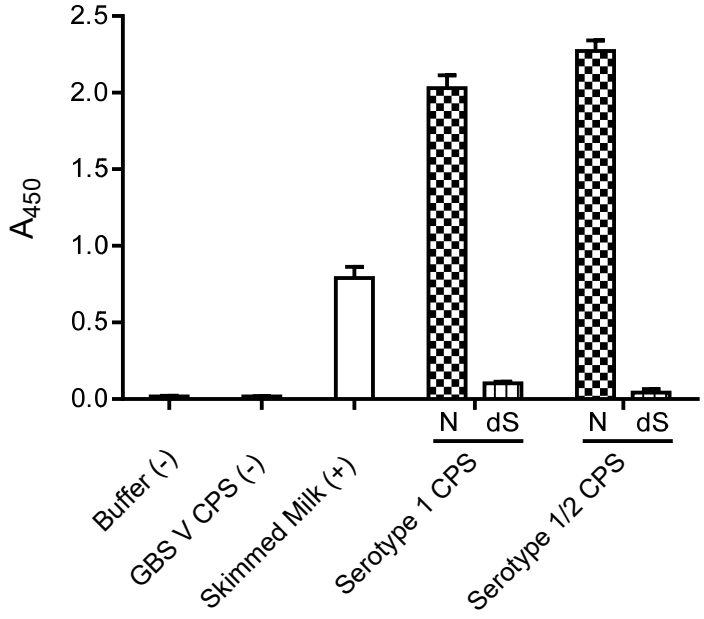


Figure 1. Enzyme-linked lectin assay (ELLA) of native and desialylated polysaccharides of *S. suis* serotypes 1 and 1/2 using *Sambucus nigra* agglutinin (SNA).

N: native CPS preparations, and dS: desialylated polysaccharide preparations per serotype. Data are shown as mean ± SEM. ELLA experiments were performed at least twice.

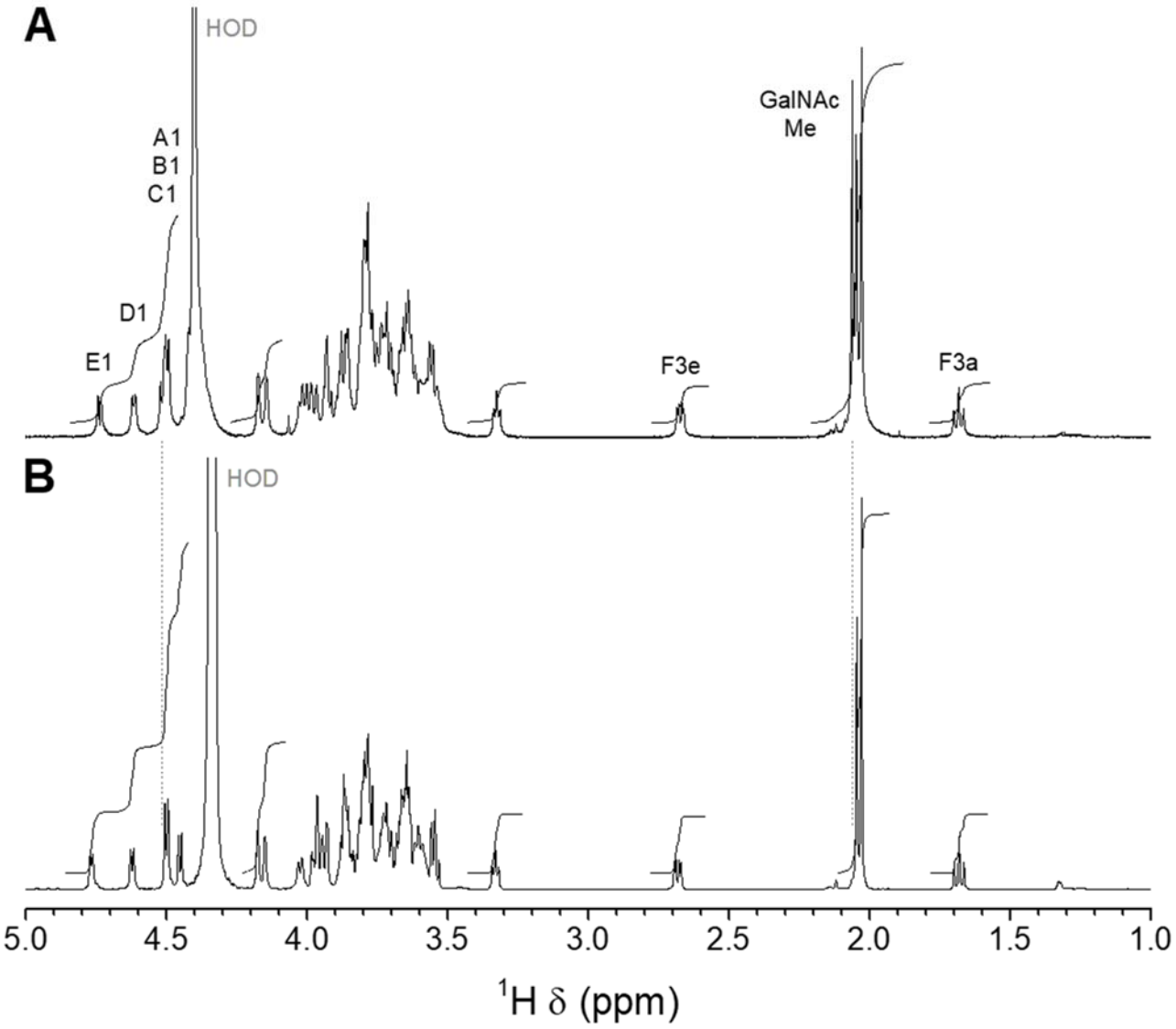


Figure 2. 700.3-MHz 1D ^{1}H NMR spectra of *S. suis* CPSs in phosphate buffer pD 8.0 in D_2O.

(A) Serotype 1, 33 mM, 70 °C. 32 K complex data points were acquired with a digital resolution of 0.21 Hz/point and processed by exponential multiplication, zero filling, Fourier transform, phase correction, and fifth-order polynomial least-squares baseline correction. (B) Serotype 14, 80 mM, 77 °C. 16 K complex data points were acquired with a digital resolution of 0.31 Hz/point and processed as above. Vertical dotted lines point to major differences between the two spectra.

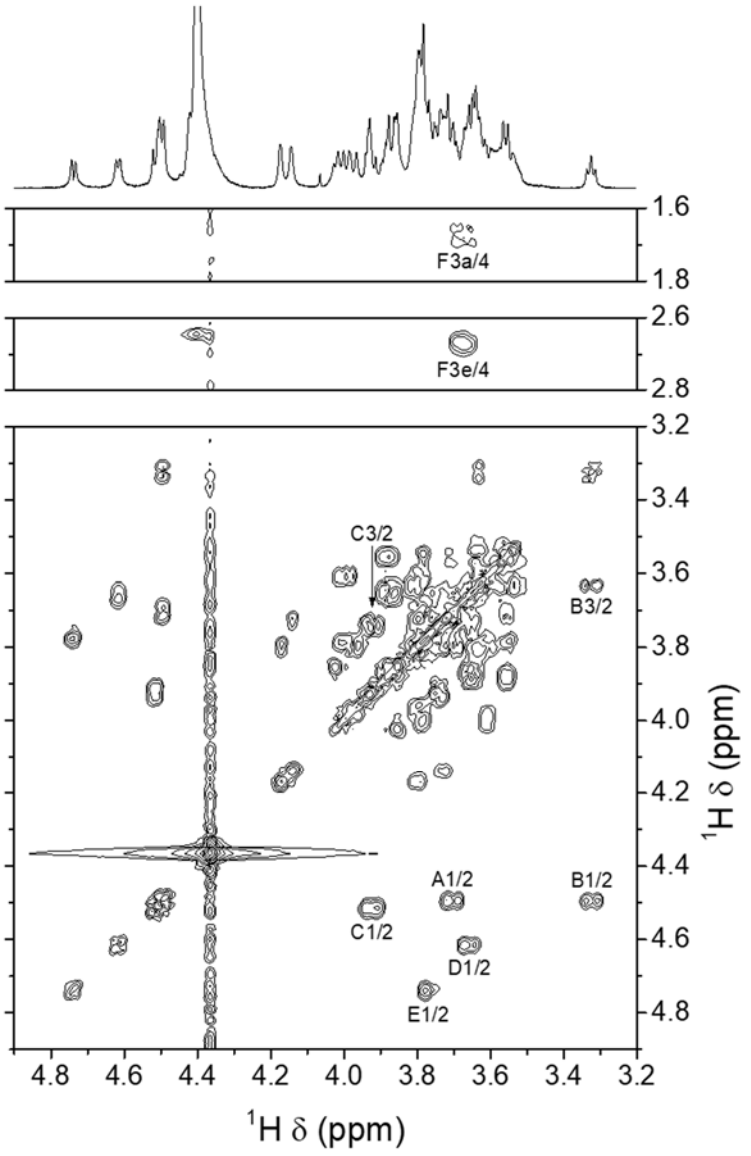


Figure 3. Portions of the 500.1-MHz ge-2D COSY spectrum of *S. suis* serotype 1 CPS in 33 mM phosphate buffer pD 8.0 in D_2O at 65 °C.

512 increments of 1 K complex data points were acquired in magnitude mode with a digital resolution of 4.9 Hz/point in the t_2 dimension and 9.8 Hz/point in the t_1 dimension. The t_2 dimension was processed by multiplication with an unshifted sinebell window function and Fourier transform, and the t_1 dimension by Zhu-Bax linear prediction to 1024 points, multiplication with an unshifted sinebell window function, Fourier transform, and magnitude calculation. The f_2 trace corresponds to the 1D spectrum (see Figure 2A).

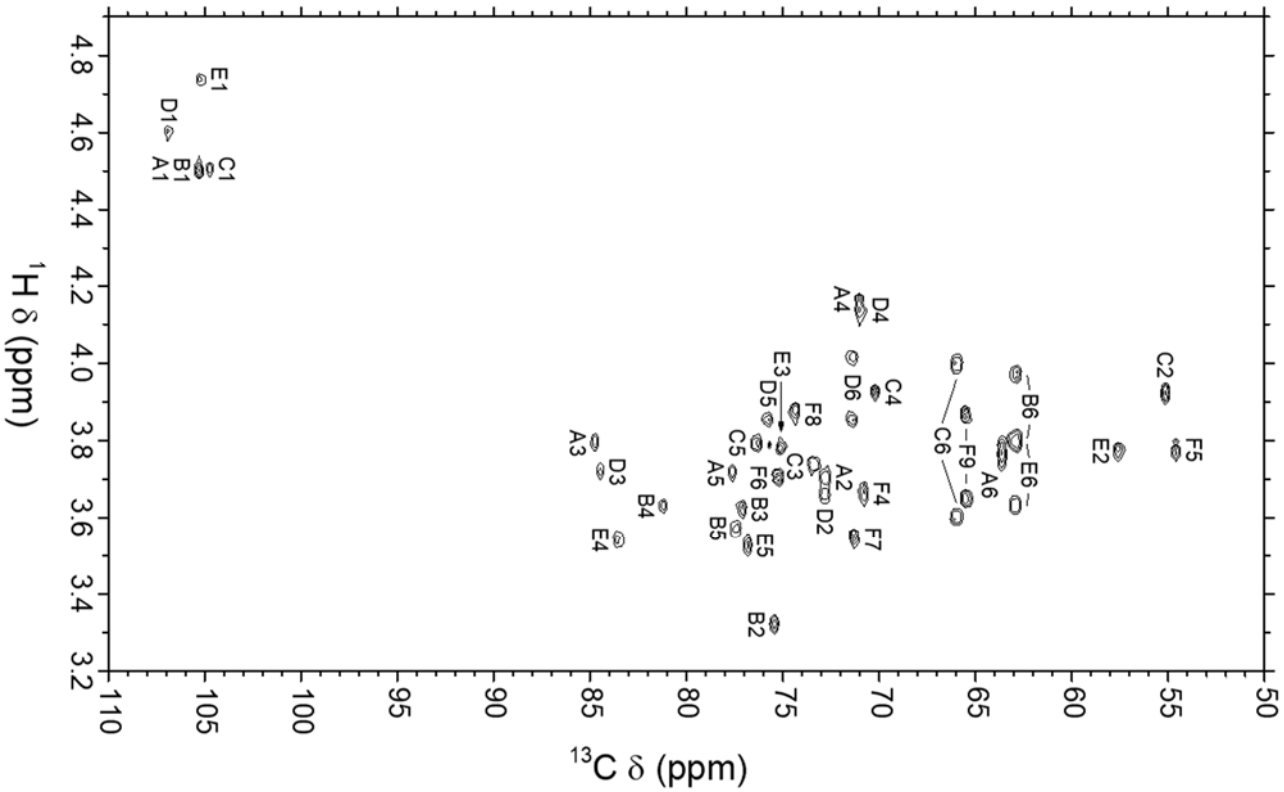


Figure 4. Portion of the 700.3-MHz ge-2D HSQC spectrum of *S. suis* serotype 1 CPS in 33 mM phosphate buffer pD 8.0 in D_2O at 70 °C.

2 × 256 increments of 819 complex data points were acquired in the States-TPPI mode with a digital resolution of 6.8 Hz/point in the t_2 dimension and 96.3 Hz/point in the t_1 dimension. The t_2 dimension was processed by multiplication with a π/2 shifted sinebell window function, zero filling, Fourier transform, and phase correction, and the t_1 dimension by Zhu-Bax linear prediction to 512 points, multiplication with a π/2 shifted sinebell window function, Fourier transform, and phase correction. Only positive contours are shown.

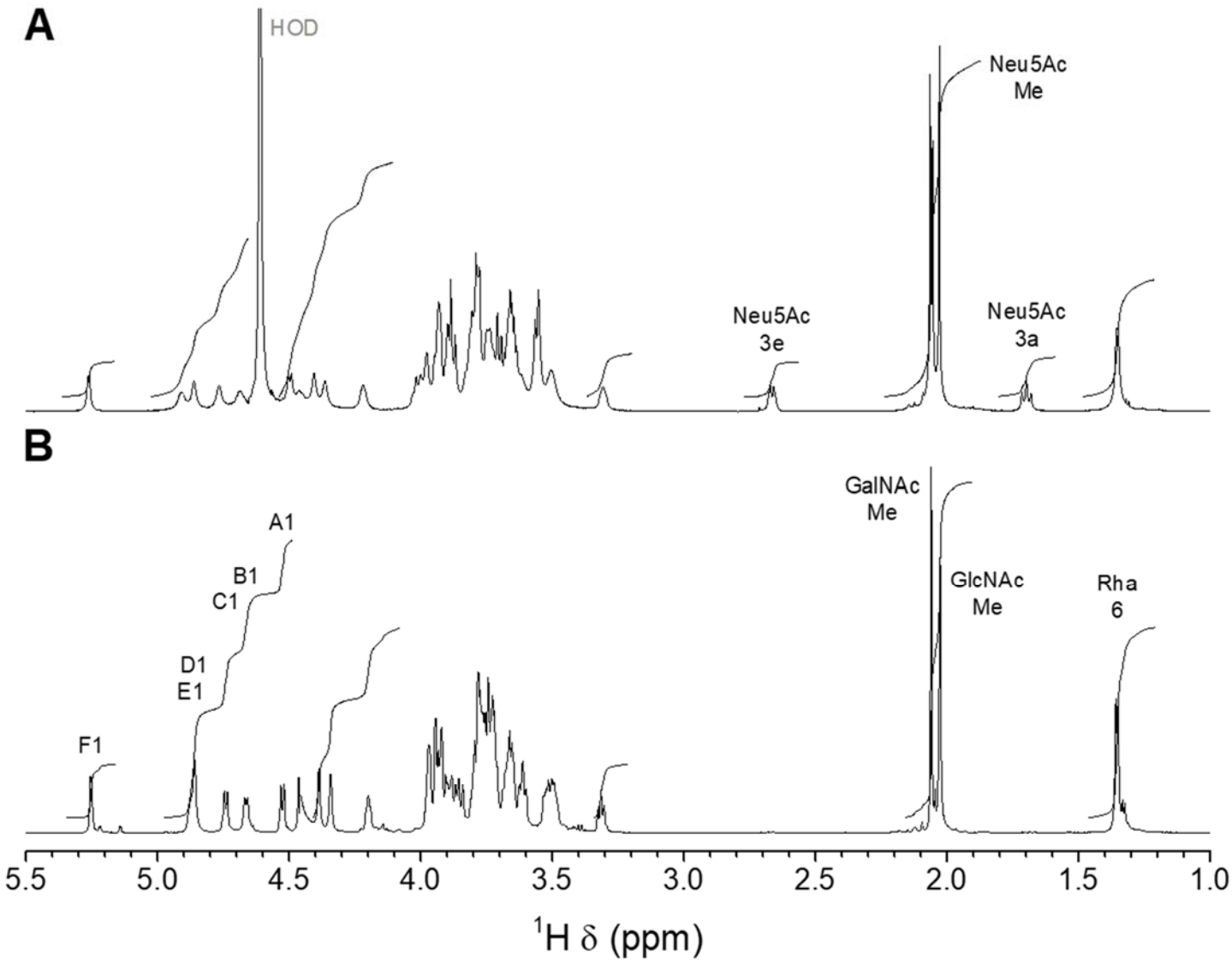


Figure 5. 700.3-MHz 1D ^{1}H NMR spectra of *S. suis* serotype 1/2 polysaccharides.

(A) Native CPS in 33 mM phosphate buffer pD 8.0 in D_2O at 42 °C. 40 K complex data points were acquired with a digital resolution of 0.18 Hz/point and processed as for Figure 2A. (B) Desialylated polysaccharide in D_2O at 56 °C. 40 K complex data points were acquired with presaturation with a digital resolution of 0.17 Hz/point and processed as above.

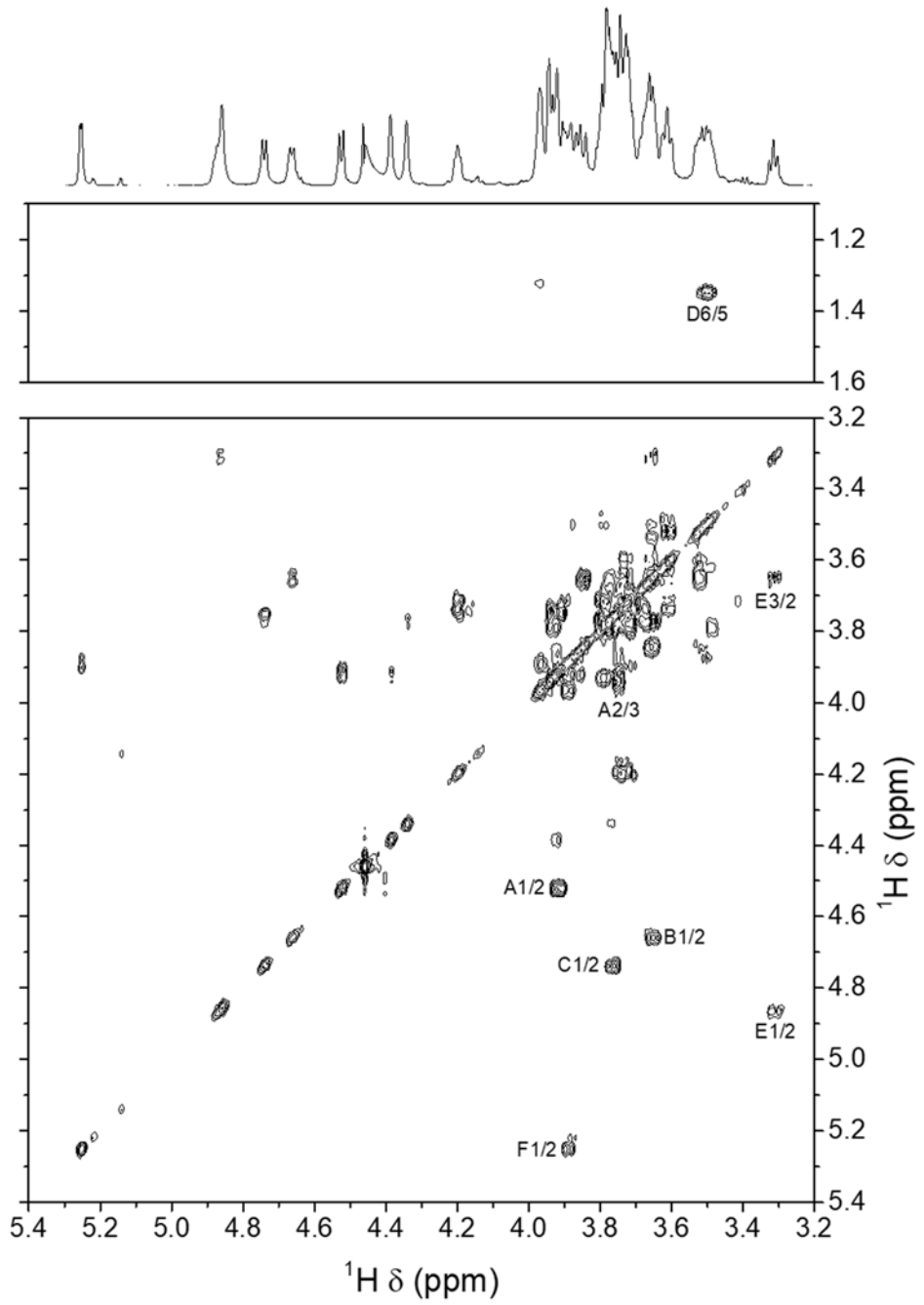


Figure 6. Portions of the 700.3-MHz ge-2D COSY spectrum of *S. suis* serotype 1/2 desialylated polysaccharide in D_2O at 56 °C.

512 increments of 2 K complex data points were acquired in magnitude mode with a digital resolution of 3.4 Hz/point in the t_2 dimension and 13.7 Hz/point in the t_1 dimension. Processing was as for Figure 3. The f_2 trace corresponds to the 1D spectrum (see Figure 5B).

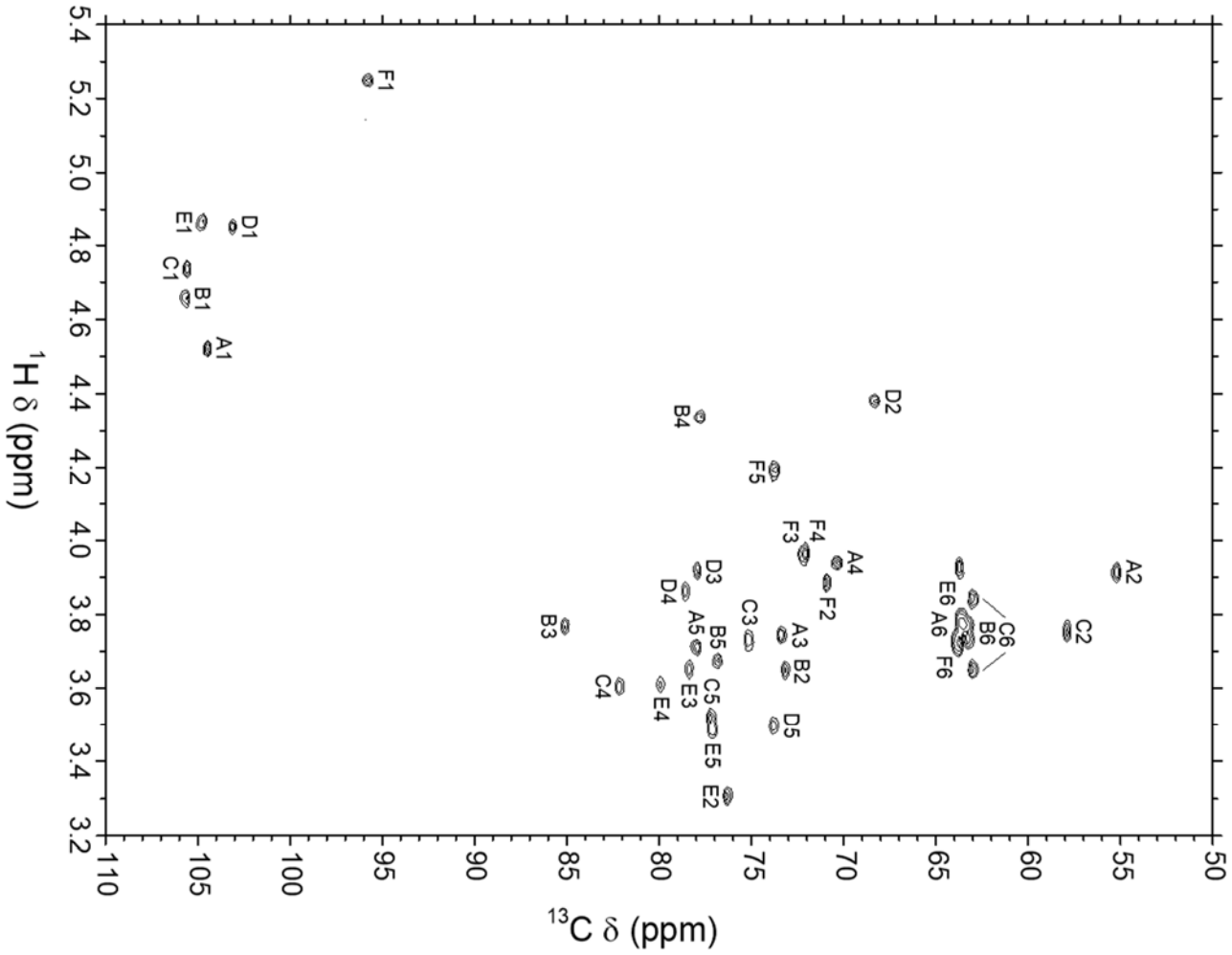


Figure 7. Portion of the 700.3-MHz ge-2D HSQC spectrum of *S. suis* serotype 1/2 desialylated polysaccharide in D_2O at 56 °C.

2 × 200 increments of 700 complex data points were acquired in the echo–antiecho mode with a digital resolution of 10.0 Hz/point in the t_2 dimension and 101.2 Hz/point in the t_1 dimension. The t_2 dimension was processed by multiplication with a π/2 shifted sinebell window function, zero filling, Fourier transform, and phase correction, and the t_1 dimension by Zhu-Bax linear prediction to 400 points, multiplication with a π/2 shifted sinebell window function, zero filling, Fourier transform, and phase correction. Only positive contours are shown.

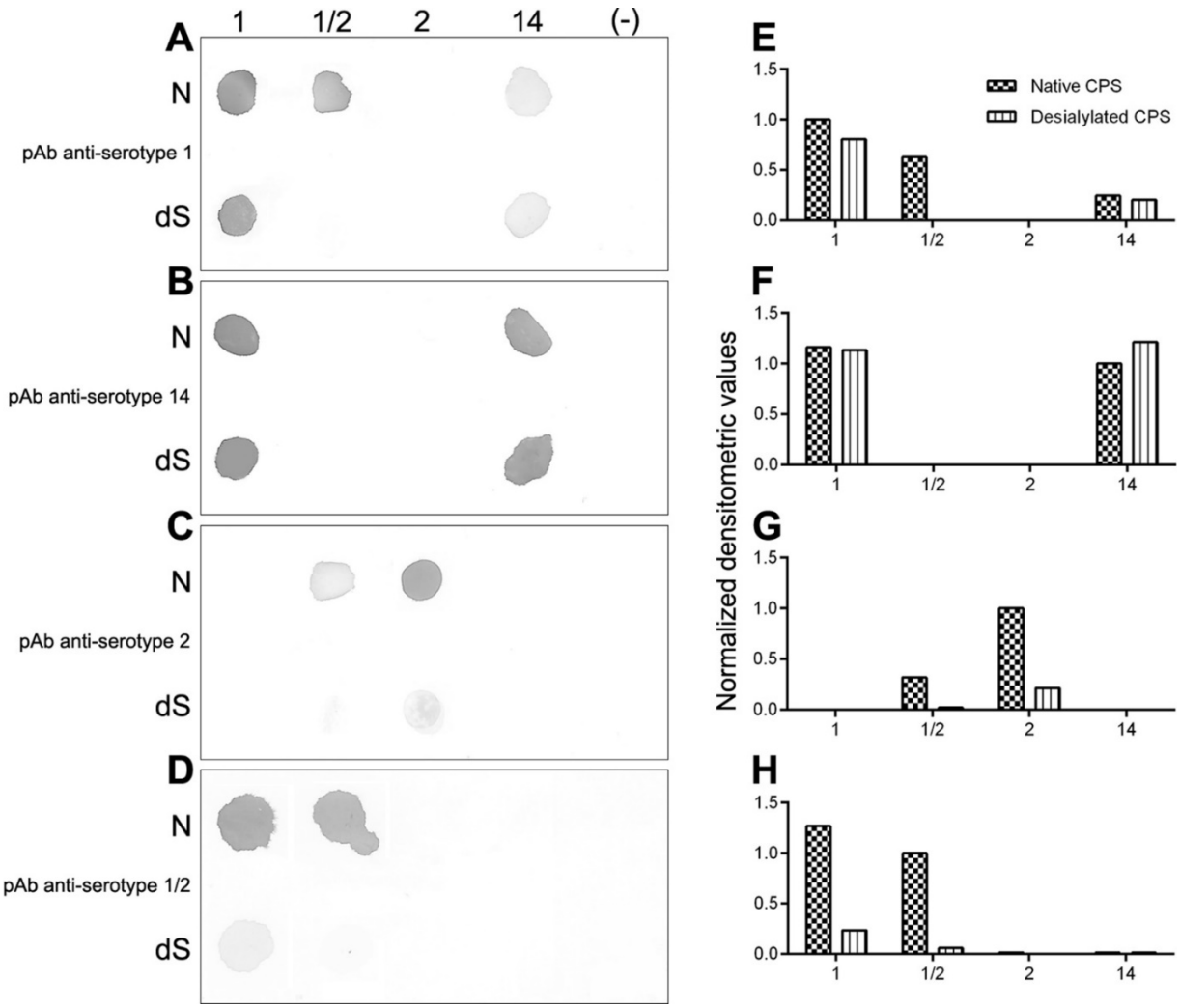


Figure 8. Serological cross-reactions.

Dot-blot analyses with 10 µl each of native CPS (N) and desialylated (dS) polysaccharide preparations from serotypes 1, 1/2, 2, and 14 of *S. suis* using different rabbit polyclonal sera (pAb): anti-serotype 1 serum (A), anti-serotype 14 serum (B), anti-serotype 2 serum (C), or anti-serotype 1/2 serum (D). Negative control (C-) is 50 mmol/L NH_4HCO_3, which was used to dissolve all CPS preparations at 1 mg/mL. Normalized integration values obtained by densitometry analysis were calculated for each dot-blot: anti-serotype 1 serum (E), anti-serotype 14 serum (F), anti-serotype 2 serum (G), and anti-serotype 1/2 serum (H).

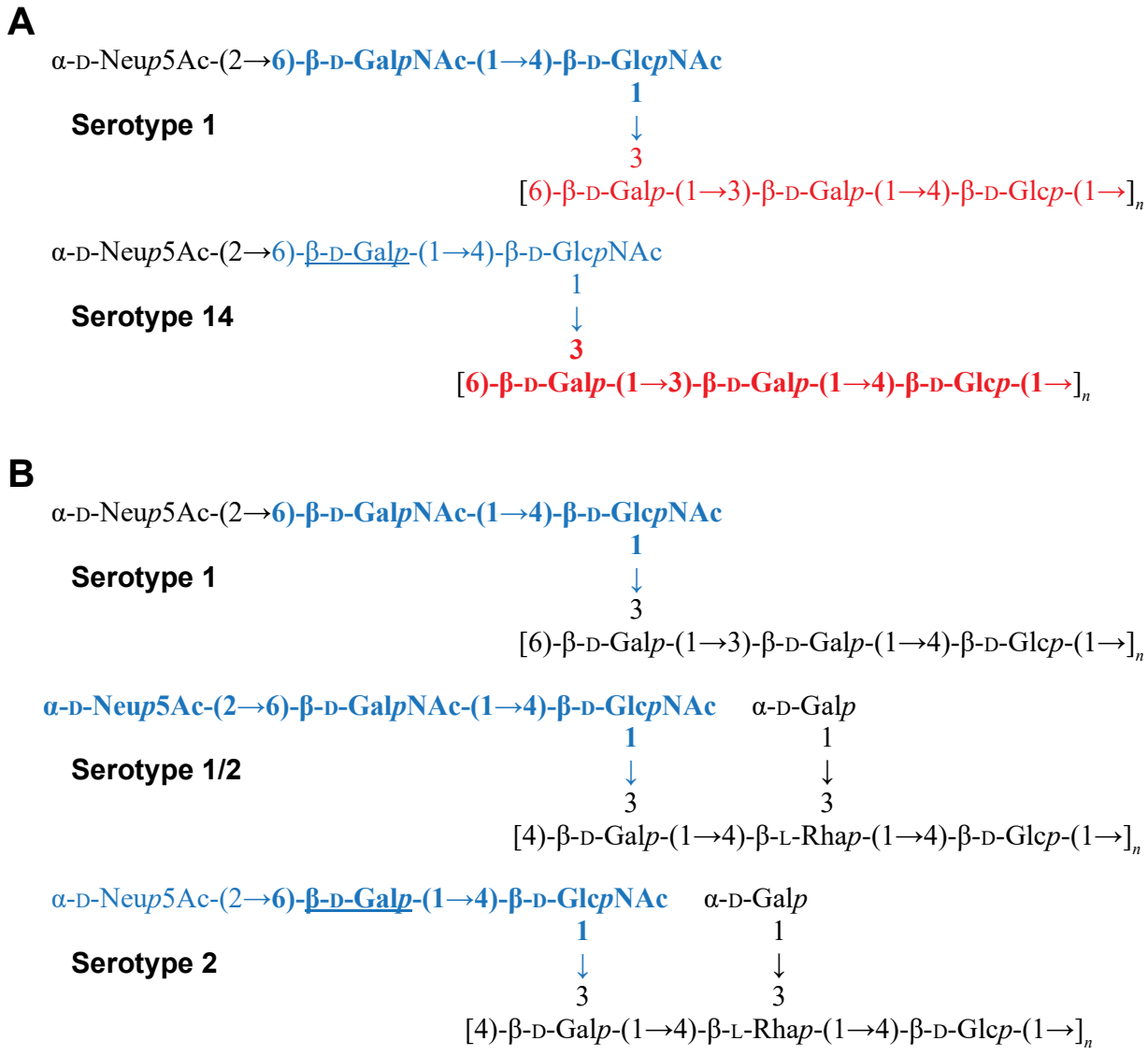


Figure 9. Proposed structural elements recognized by type-specific antisera implicated in the serological cross-reactions between serotypes 1 and 14 (A), and serotype 1/2 with serotypes 1 and 2 (B).

Colored elements represent those sequences probably involved in cross-reactions. In red are indicated the recognized structures from the backbone sequence, whereas in blue are the structures from the side chains. In bold are highlighted the structures yielding the dominant antibody population. An underline is used to illustrate structural elements for which the cross-reaction is weaker.

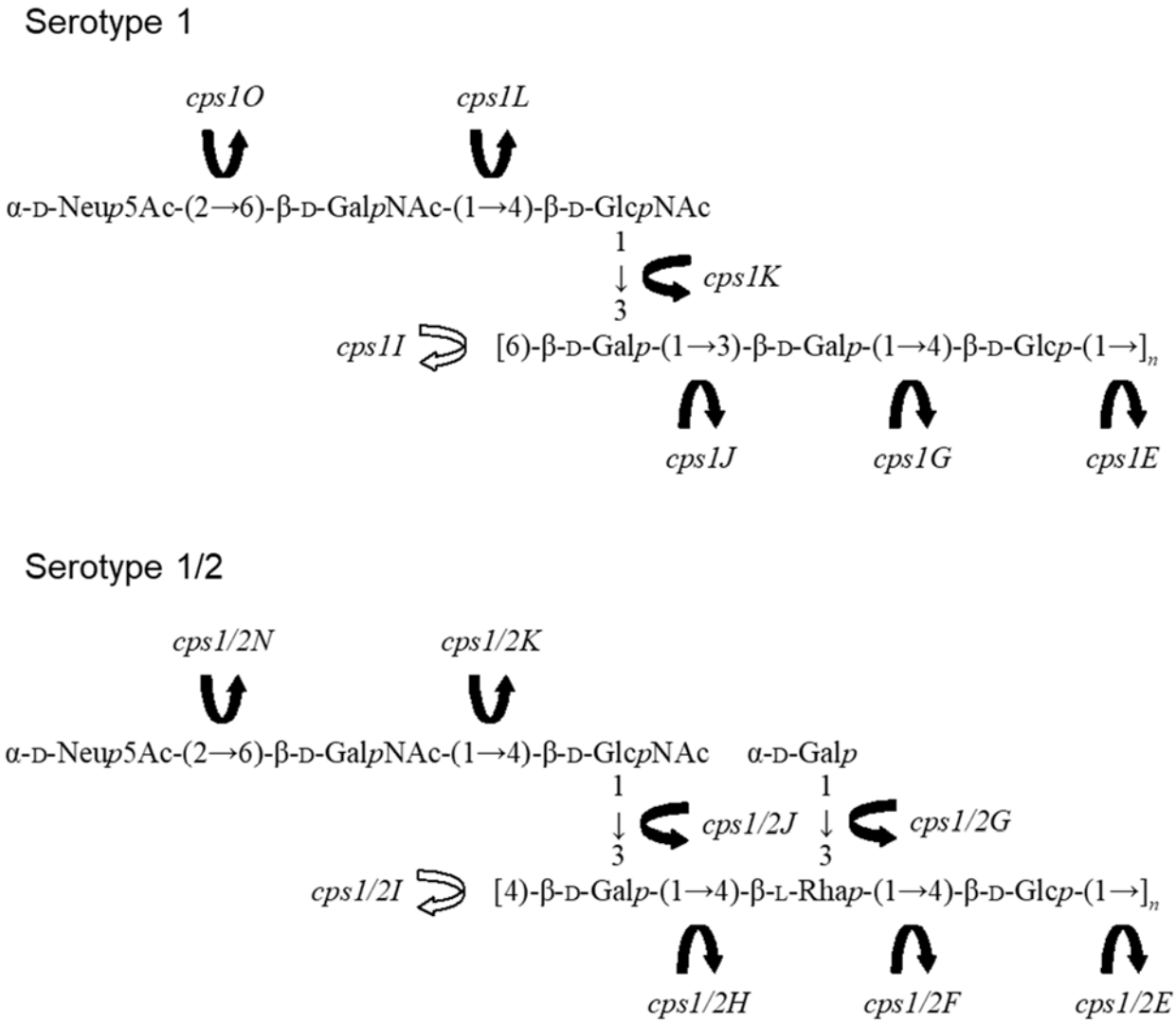


Figure 10. Tentative correlation between structure and genes encoding enzymes responsible for the biosynthesis of *S. suis* CPSs.

Black arrows: glycosyltransferases; white arrows: polymerases.

Supporting information

Supplementary material is available with the article through the journal Web site at http://www.jbc.org/content/291/16/8387/suppl/DC1.

Table S1. ^{13}C NMR chemical shift differences (ppm) between ring carbons of *N*-acetylgalactosamine residues and of corresponding galactose residues in CPSs and methyl glycosides.

Table S2. Glycosyltransferase and Wzy polymerase genes in the *cps* locus of *S. suis* serotypes 1, 1/2, 2, and 14.

Figure S1. Regions of the 176.1 MHz 1D ^{13}C NMR spectra of *S. suis* serotype 1 CPS in 33 mM phosphate buffer pD 8.0 in D_2O at 70 °C.

Figure S2. Portions of the 500.1-MHz 2D TOCSY spectrum of *S. suis* serotype 1 CPS in 33 mM phosphate buffer pD 8.0 in D_2O at 65 °C.

Figure S3. Portion of the 500.1 MHz ge-2D T-ROESY spectrum of *S. suis* serotype 1 CPS in 33 mM phosphate buffer pD 8.0 in D_2O at 65 °C.

Figure S4. Portion of the 700.3 MHz ge-2D HSQC–TOCSY spectrum of *S. suis* serotype 1 CPS was obtained in 33 mM phosphate buffer pD 8.0 in D_2O at 70 °C.

Figure S5. Portions of the 700.3 MHz HMBC spectrum.

Figure S6. Regions of the 176.1 MHz 1D ^{13}C NMR spectra of *S. suis* serotype 1/2 desialylated polysaccharide in D_2O at 56 °C.

Figure S7. Portions of the 700.3-MHz 2D TOCSY spectrum of *S. suis* serotype 1/2 desialylated polysaccharide in D_2O at 56 °C.

Figure S8. Portion of the 700.3 MHz 2D ROESY spectrum of *S. suis* serotype 1/2 desialylated polysaccharide in D_2O at 56 °C.

Figure S9. Portion of the 700.3 MHz ge-2D HSQC–TOCSY spectrum of *S. suis* serotype 1/2 desialylated polysaccharide in D_2O at 56 °C.

Figure S10. Portions of the 700.3 MHz HMBC spectrum.

Figure S11. Portion of the 700.3 MHz ge-2D HSQC spectrum of *S. suis* serotype 1/2 native CPS in 33 mM phosphate buffer pD 8.0 in D_2O at 42 °C.

Figure S12. A multiple amino acid sequence alignment of proteins encoded by *cps* genes performed using ClustalW2.1 (default parameters).

ANNEXES - ARTICLE XI

Structure determination of *Streptococcus suis* serotype 9 capsular polysaccharide and assignment of functions of the *cps* locus genes involved in its biosynthesis

---

Evgueny Vinogradov [1], **Guillaume Goyette-Desjardins** [2], Masatoshi Okura [3], Daisuke Takamatsu [3,4], Marcelo Gottschalk [5], and Mariela Segura [2]

[1] National Research Council, Ottawa, Ontario, Canada;

[2] Laboratory of Immunology, Faculty of Veterinary Medicine, University of Montreal, St-Hyacinthe, Quebec, Canada;

[3] Bacterial and Parasitic Diseases Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan;

[4] The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Gifu, Japan;

[5] Laboratory of research on *Streptococcus suis*, Faculty of Veterinary Medicine, University of Montreal, St-Hyacinthe, Quebec, Canada.

Publié dans *Carbohydrate Research*. 2016. 433:25-30.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé partiellement aux expériences de purification (30%) ainsi qu'à l'analyse des résultats et à la rédaction de l'article.

Abstract

Streptococcus suis serotype 9 is the most prevalent *S. suis* serotype in several European countries. In spite of its pathogenicity for pigs and increasing zoonotic potential, limited information is available on this serotype. Here we determined for the first time the chemical composition and structure of serotype 9 capsular polysaccharide (CPS), a major bacterial virulence factor and the antigen at the origin of *S. suis* classification into serotypes. Chemical and spectroscopic data gave the repeating unit sequence: [3)Glcol-6-*P*-3-[D-Gal(α1-2)]D-Gal(β1-3)D-Sug(β1-3)L-Rha(α1-)]n. Compared to previously characterized *S. suis* CPSs (serotypes 1, 1/2, 2 and 14), serotype 9 CPS does not contain sialic acid but contains a labile 4-keto sugar (2-acetamido-2,6-dideoxy-β-D-*xylo*-hexopyranos-4-ulose), one particular feature of this serotype. A correlation between *S. suis* serotype 9 CPS sequence and genes of this serotype *cps* locus encoding putative glycosyltransferases and polymerase responsible for the biosynthesis of the repeating unit was tentatively established. Knowledge of CPS structure and composition will contribute to better dissect the role of this bacterial component in the pathogenesis of *S. suis* serotype 9.

1. Introduction

Streptococcus suis ranks among the five most important health challenges of pigs worldwide. It is associated with numerous diseases, such as meningitis, septicemia, arthritis and endocarditis in both traditional and modern intensive swine operations. *S. suis* is also an emerging zoonotic agent in humans and thus considered a public health threat, especially for people working in the swine/pork industry. The natural habitat of *S. suis* is the upper respiratory tract, more particularly the tonsils and nasal cavities, but also the genital and digestive tracts of pigs of all ages. Transmission of the infection between herds usually occurs by the movement of healthy carrier animals harboring virulent strains. Sows infect their own piglets during the birth process and probably also through the respiratory route. In fact, horizontal transmission seems to be significant, especially in the presence of clinical signs, with a considerable higher number of bacteria in the environment that would increase transmission either by aerosol or direct contact. Pig carrier rate of *S. suis* is close to 100%, although the proportion of animals colonized by virulent strains is lower [1].

S. suis isolates express a "capsular polysaccharide" (CPS) which is considered one of the major bacterial virulence factor [2–4]. Originally, 35 serotypes were described and the structure of the CPS defines the serotype. Recent molecular analyses have led to the proposed reclassification of six of those serotypes as other species than *S. suis*, namely serotypes 20, 22, 26, 32, 33, and 34; and the discovery of novel cps loci [5–7]. Yet, most *S. suis* organisms isolated from diseased pigs belong to a limited number of serotypes. Serotype 2 has been worldwide considered the most prevalent and virulent *S. suis* type [8]. However, there is a clear geographical effect on the distribution of serotypes, and recent epidemiological studies indicates that serotype 9 is the most prevalent serotype in several European countries, such as Spain and the Netherlands [8].

As mentioned above, *S. suis* is also an emerging zoonotic agent, being responsible for septicemia with or without septic shock, meningitis and other less common infections usually related to generalized septicemia. Albeit well-known in veterinary medicine, its significance has been neglected in human medicine. Infections in humans were considered sporadic in

people working with pigs or pork-derived products. Yet, recent epidemiological data from Asia, including important and deadly human outbreaks, has changed the perspective of the threat posed by this pathogen to human health. While in Western countries infections in humans are considered an occupational disease, in some Asian countries, the general population is at risk. Different from pigs, the main route of entry of *S. suis* in humans is through skin contact with contaminated animals, carcasses or meat. However, this situation seems to be different in some Asian countries where the oral route is taken into consideration after ingestion of contaminated raw pork products as part of traditional dishes. Serotype 2 is the most prevalent in humans, but sporadic cases with other serotypes have been also described [8]. A recent report of a human case due to serotype 9 in Thailand suggests a high zoonotic potential for this serotype as well [9].

The CPS is an important virulence factor at the interface of the bacteria and the host, contributing to the modulation of bacterial colonization, systemic dissemination and bacterial persistence. The CPS regulates the innate and the adaptive immune response of the infected host and is the target of protective antibodies. Finally, it is the antigen at the base of serological and molecular diagnostic and serotyping [8,10]. Due to its importance, ongoing research yielded complete structural determination for the CPSs of *S. suis* serotype 2 [11], serotype 14 [12], and serotypes 1 & 1/2 [13]. A sialic acid-containing side chain was found as a common feature of all these four serotypes. Besides, lectin-based studies and cps locus genetic analyses predicted the presence of this sugar in serotypes 6, 13, 16 and 27 as well [14,15]. Albeit serotype 9 does not belong to the *S. suis* sialylated-CPS group, it presents a high virulence and zoonotic potential. Thus, in this work, we describe for the first time the purification and the primary structure of the CPS polymer for *S. suis* serotype 9.

2. Material and methods

2.1. Capsular polysaccharide production and purification

S. suis serotype 9 field strain 1273590 was used in this study (isolated from a diseased pig in Quebec, Canada - our collection). For the CPS production, 6 L of Todd-Hewitt Broth was inoculated and grown overnight as previously described [12]. Two methods of extraction

were compared. Method A was based in previous publications [11,12]. Briefly, bacterial cells from the 6 L culture were pelleted by centrifugation at 10 000g for 40 min, suspended by repeated pipetting in a buffer containing 33 mmol/L phosphate and 145 mmol/L NaCl pH 8.0, and chilled. The bacterial suspension was autoclaved at 121 °C for 15 min. The supernatant containing the crude capsule was recovered by centrifugation at 9000g for 50 min. Extraction with an equal volume of chloroform eliminated lipids, whereas nucleic acids were removed by precipitation by adding CaCl2 to 0.1 mol/L and ethanol to 25% v/v, and then centrifuged at 7200g for 30 min at room temperature. The material was then concentrated by precipitating in ethanol 80% v/v. Pellets were dissolved in 50 mmol/L NH4HCO3, dialyzed against the same solution for 48 h with a Spectra/Por 2 membrane (Spectrum Laboratories) MWCO 12–14 kDa, and freeze dried. The CPS was further purified by gel filtration chromatography on a XK-26-100 column filled with Sephacryl S-400 HR (GE Healthcare, Uppsala, Sweden) eluted with 50 mmol/L NH4HCO3 at a flow rate of 1.3 mL/min, using an ÄKTA Purifier 10 system (GE Healthcare), including a UV-900 Monitor, and equipped with a Knauer Smartline 2300 RI Detector (Knauer, Berlin, Germany) connected to the system via an AD-900 Analog/Digital Converter (GE Healthcare). Fractions were collected and assayed for CPS by dot enzyme-linked immunosorbent assay (dot-ELISA) with an anti-*S. suis* serotype 9 rabbit polyclonal antibody (1/500) as the primary antibody and horseradish-peroxidase-conjugated (HRP-conjugated) goat anti-rabbit secondary antibody (1/3000). Fractions giving a positive RI signal and response with antibodies but no absorption at 280 and 254 nm were pooled and freeze dried. The purified material was dissolved in water, dialyzed against ddH2O for 48 h at 4 °C, and finally freeze dried.

For Method B, bacterial pellet from the 6 L culture was resuspended in deionized water (ddH2O) and washed 2 times. The bacteria were killed by heating at 60 °C for 45 min, which have been confirmed by the absence of growth on blood agar plates. Finally, the content of the tubes were lyophilized for 72 h. Then, bacterial cells were stirred in water (3 g dry cells in 100 mL of water) at room temperature overnight, cells removed by centrifugation at 12000g 20 min, solution dialyzed, dried, dissolved in 2 mL of water, insoluble material removed by centrifugation (12000g, 10 min), solution separated on Biogel P10 column (2.5 × 60 cm; Bio Rad, Hercules, CA) in 1% AcOH. Broad peak starting at void volume was collected in 2

fractions. The eluent was turbid and viscous after this step and was spun 120000 g for 1 h. After small amount of precipitate was discarded, solution became clear, colorless and not viscous. Both fractions from gel chromatography contained the same polysaccharide, second (lower molecular mass) fraction contained more impurities as indicated by nuclear magnetic resonance (NMR) (data not shown). Extraction of the remaining water-washed cells by 45% phenol at 70 °C produced no polysaccharides.

2.2. NMR spectroscopy

NMR experiments were carried out on a Bruker AVANCE III 600 MHz (1H) spectrometer with 5 mm Z-gradient probe with acetone internal reference (2.225 ppm for 1H and 31.45 ppm for 13C) using standard pulse sequences cosygpprqf (gCOSY), mlevphpr (TOCSY) (mixing time 120 ms), roesyphpr (ROESY) (mixing time 500 ms), hsqcedetgp (HSQC), hsqcetgpml (HSQC-TOCSY) (80 ms TOCSY delay) and hmbcgplpndqf (HMBC) (100 ms long range transfer delay). Resolution was kept <3 Hz/pt in F2 in H-H correlations and <5 Hz/pt in F2 of H-C correlations. Number of points in F2 set so to have AQ about 0.8 s for H-H correlations, 0.24 s for C-H HSQC. Number of points in F1 was 1/4 of F2. The spectra were processed and analyzed using the Bruker Topspin 2.1 program.

2.3. Determination of neutral and amino sugars as alditol acetates

Monosaccharides were detected as reduced and acetylated derivatives (alditol acetates). Polysaccharide sample (0.2 mg) with inositol internal standard was dephosphorylated with 48% hydrofluoric acid (10 μL, 30 min, 30 °C), dried by air stream, and hydrolyzed with 3 M trifluoroacetic acid (120 °C, 3 h). The sample was then dried, reduced with NaBD4, and reagent destroyed with 0.5 mL of AcOH. The obtained solution dried under the stream of air, dried twice with addition of MeOH (1 mL), acetylated with 0.4 mL Ac2O - 0.4 mL pyridine for 30 min at 100 °C, dried, and finally analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) (Varian Saturn 2000 ion-trap instrument, capillary column DB-17, 160–260 °C by 4°/min).

2.4. Absolute configurations

For the determination of absolute configuration, the polysaccharide (0.3 mg) was dephosphorylated with 48% HF (10 µL, 30 min, 30 °C), dried by air stream, (R)- or (RS)-2-octanol (0.2 mL) and acetyl chloride (20 µL) were added at room temperature, heated at 100 °C for 2 h, dried by air stream, acetylated (0.2 mL Ac2O-0.2 mL pyridine, 100 °C, 30 min), dried, and finally analyzed by GC-MS as described above.

2.5. Comparison of cps genes

The nucleic acid and amino acid sequences of cps genes were compared using blastn, blastp and tblastx algorithms at National Center for Biotechnology Information (NCBI) network service (http://blast.ncbi.nlm.nih.gov/) [16]. The Artemis Comparison Tool (ACT) [17] was used to visualize the comparison data between two cps gene clusters (bit-scores above 50 and E-values lower than 1e-8).

3. Results and discussion

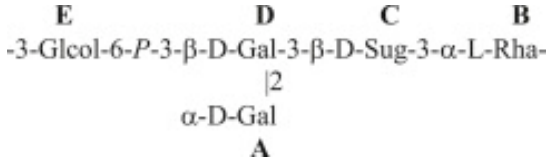
3.1. Capsular polysaccharide production and purification

Using Method A, from 6 L of fermentation broth in two different experiments, 61–66 mg of CPS was obtained after purification by gel filtration. Unexpectedly, the purified serotype CPS by gel filtration chromatography yielded two major CPS peaks exempt of protein detection (UV 280 nm signal). Analysis of the two CPS peaks showed same structures and the presence of at least 3 isomers of 2-acetamido-2,6-dideoxy-hexopyranos-4-ulose (‘‘4-keto’’ sugar; data not shown), suggesting that Method A used for CPS purification (namely by heating at 121 °C the bacterial suspension in alkaline conditions) has led to modification of the structure by isomerization of 4-keto sugar. This led to complicated spectra (data not shown). In order to improve purification and analysis, we used milder CPS extraction conditions (Method B). The polysaccharide was simply extracted from dried cells by water at room temperature, suggesting that polysaccharide was not covalently bound to the cells, and probably some of it was released into culture medium.

3.2. Structure determination and NMR

S. suis serotype 9 CPS structure and composition was determined for the first time. Polysaccharide (purified by Method B) was analyzed by 2D NMR using COSY, TOCSY, ROESY, 1H-13C HSQC, HMBC, HSQC-TOCSY, and 1H-31P HMQC experiments. Monosaccharides were identified by COSY, TOCSY and NOESY cross peak patterns and 13C NMR chemical shifts (Fig. 1, Table 1). Amino group location was concluded from high field signal position of aminated carbons (CH at 45–60 ppm). Spectra contained signals of tetrasaccharide repeating units made from α-Gal, β-Gal, α-Rha, and 2-acetamido-2,6-dideoxy-β-xylo-hexopyranos-4-ulose (Sug, unit C), one phosphate and one glucitol residue per repeating unit. All sugars were in pyranose form, as followed from the absence of low-field 13C signals characteristic for furanose form of monosaccharides. All sugars had NMR signal positions and coupling constants in agreement with the presented structure. Relatively rare Sug C had no H-4 and had J1,2 8 Hz and J2,3 10 Hz, which indicated β-xylo-configuration assuming 4C1 conformation. Position of its C-4 signal was found from HMBC spectrum. The obtained chemical shift of C-4 of the ‘‘4-keto’’ sugar indicated that it was a hydrated form. No attempts was made to find correct numbering in glucitol residue, thus it can be presented as 3,6-substituted, as shown on the structure below, or 1,4-substituted. Rha, Gal and glucitol were identified by GC-MS using NaBD4 reduction, which allowed to distinguish glucitol from glucose. L-Rha and D-Gal absolute configurations were determined by GC-MS of 2-octyl glycosides acetates with optically pure 2-octanol.

The sequence of the monosaccharides was determined from the following NOE and HMBC inter-residual correlations: A1:D2 (and NOE A1:D1w); B1:E3; C1:B3; D1:C3. Protons D3 and E6 correlated with the same phosphate signal at 0.4 ppm, indicating E-6-P-3-D linkage, as shown:



Absolute D-configuration of the Sug was found by calculation of 13C NMR shifts for the pairs β-D-Sug-3-α-L-Rha and β-L-Sug-3-α-L-Rha, which showed good agreement with experiment for D-L pair and quite poor one for L-L combination [18].

Spectra contained minor series of the signals that can be attributed to the same repeating units containing isomerization products of the Sug. These signals indicate degradation of the polysaccharide during NMR measurement at 50 °C and became visibly more intense after 48 h in spectrometer. Most probably transformation of the Sug proceeds via keto-enol tautomerization and subsequent inversion of C-3 and/or C-5 carbons. Extraction of the polysaccharide in autoclave at 120 °C leads to nearly complete disappearance of the xylo-isomer and formation of two main products with the same skeleton but inverted configurations.

Altogether our results showed that *S. suis* serotype 9 CPS structure and composition is different from previously identified *S. suis* CPSs (serotypes 1, 1/2, 2 and 14). In summary, the polysaccharide had regular structure and was composed of 4 different monosaccharides with a side chain formed by single Gal residue. One particular feature is the presence of the 4-keto Sug. This monosaccharide is usually produced by 4,6-dehydration of UDP-α-GlcNAc to give UDP-2-acetamido-2,6-dideoxy-α-xylo-hexopyranos-4-ulose [19]. This way of biosynthesis ensures its D-configuration, which is normally found in Nature. A recent list of structures of O-specific and capsule polysaccharides containing monosaccharides of this class was reported by Kokoulin et al. [20]. Surprisingly in this publication authors found the monosaccharide to have L-configuration in the case of the marine bacterium Litorimonas taeanensis, which was not explained. Among related microorganisms, the presence of 4-keto Sug was also found in Streptococcus pneumoniae type 5 CPS [21] and in Staphylococcus saprophyticus CPS (ATCC strain 15305) [22]. It has been reported that presence of 4-keto Sug accounts for the lability of S. pneumoniae type 5 CPS towards alkali [21], and thus explain the difficulties encountered when using Method A to purify *S. suis* serotype 9 CPS, including isomerization. Similar situation was observed when analyzing S. saprophyticus CPS [22] (E. Vinogradov, unpublished observations).

3.3. Assignment of functions of the cps locus genes involved in its biosynthesis

Genome sequences of *S. suis* serotype 9 reference strain and a Chinese field strain are available [GenBank accession no. NZ_ALKY00000000.1 and CP002644.1, respectively [23,24]], and the serotype 9 *cps* operon was previously characterized [GenBank accession no. BR001006] [15,25,26]. Based on sequence homologies a correlation was tentatively established between the CPS structure determined in this study and the genes encoding putative glycosyltransferases and a polymerase, which are clustered in the *S. suis* serotype 9 *cps* locus (Fig. 2 and Table 2). Overall structure and nucleotide sequence of *cps* loci are not similar between *S. saprophyticus* ATCC 15305 and *S. suis* serotype 9 reference strain. However, Cps9C, Cps9D, Cps9E, and Cps9F showed some amino acid sequence similarity to proteins encoded in the *cps* locus of *S. saprophyticus* ATCC 15305 (Fig. 3 and Table 2). In addition, Cps9A, Cps9L, and Cps9M also showed some amino acid sequence similarity to proteins encoded in other regions of *S. saprophyticus* ATCC 15305 chromosome (i.e., not in the cps locus) (Table 2). Cps9E is similar to CapD (SSP0063) of *S. saprophyticus* ATCC 15305. According to Park et al. [22], putative function of *S. saprophyticus* CapD is UDP-GlcNAc 4,6-dehydrase. Because 4-keto Sug is usually produced by 4,6-dehydration of UDP-α-GlcNAc, Cps9E is considered to be involved in 4-keto Sug synthesis. Cps9F is similar to initial glycosyltransferase WciI of *S. pneumoniae* serotypes 4, 5, 12A, 12F and 45 [27]. Because initial glycosyltransferases are usually encoded as the first glycosyltransferase in *cps* loci, Cps9F is also considered to be the initial glycosyltransferase. Initial sugar of *S. pneumoniae* serotype 5 CPS is 2-acetamido-2,6-dideoxy-β-*xylo*-hexopyranos-4-ulose, like in *S. suis* serotype 9 CPS. Thus it might initiate polysaccharide chain polymerization here as well, and Cps9F may catalyze the transfer of the first sugar to a membrane-associated lipid carrier. Cps9G is similar to glycosyltransferases WciV of *S. pneumoniae* serotype 18F, 18A, 18B and 18C [27]. In this work, wciV was depicted as the fourth glycosyltransferase gene in these *S. pneumoniae* serotypes. Given that glycosyltransferases work in the order of genes in the cps locus, WciV may transfer Gal to Glc (18F, 18B, 18C) or to GlcNAc (18A) [27]. Based on the hypothesis that the initial sugar of CPS9 repeat unit is really 4-keto Sug, Cps9G may transfer Gal to 4-keto Sug as the second glycosyltransferase. Cps9H is similar to a glycosyltransferase WcxS of *S. pneumoniae* serotype 45. As described below, Cps9I is predicted to transfer Glcol to Gal, so this third glycosyltransferase may be involved in side

chain (Gal-Gal) formation (Fig. 2). Indeed, *S. pneumoniae* serotype 45 CPS has similar side chain (Gal(α1-6)Gal) [27]. Cps9I is similar to LicD-family phosphotransferases WcrB of S. pneumoniae serotype 10F and 10A, and WcyR of *S. pneumoniae* serotype 45 (Table 2). Bentley et al. reported wcrB as the third glycosyltransferase gene in *S. pneumoniae* serotype 10F and 10A cps loci [27]. Given that glycosyltransferases work in the order of genes in the *cps* locus, WcrB is considered to be involved in the linkage of Ribtol (5-P-6) Gal or Ribtol (5-P-5) Gal. Because of two branches in the *S. pneumoniae* serotype 45 CPS unit, it is difficult to predict WcyR function in serotype 45. However, because the only linkage including sugar phosphate is Glycerol-(1-P-6)-GalNAc in this serotype [27], WcyR of serotype 45 may be involved in this linkage. Considering that Cps9I is a homologue of WcrB and WcyR, it may catalyze the formation of Glcol (6-P-3) Gal in *S. suis* CPS9 structure (Fig. 2). Cps9K is similar to glycosyltransferases WcyS of *S. pneumoniae* serotype 27, 32F and 32A (Table 2). If the above predictions are correct, Cps9K may catalyze the transfer of the putative last sugar (Rha) to Glcol (Fig. 2). The presence of Rha (α1-P-2) Glc linkage in *S. pneumoniae* serotype 32F and 32A might support this prediction [27]. Cps9L and Cps9M are similar to Mnp1 and Mnp2 related to CDP-D-mannitol biosynthesis pathway of *S. pneumoniae* serotype 35A, respectively. Because mannitol is an isomer of glucitol, Cps9L and Cps9M may be involved in glucitol biosynthesis (Table 2).

4. Conclusion

This study provides the basis for further studies on important structural elements recognized by anti-CPS specific antibodies, and thus improve our understating of the serological reactions leading to *S. suis* serotype classification. The CPS is a proven major virulent determinant for *S. suis* serotypes 2 and 14, both harboring a terminal sialic acid in the CPS lateral chain [2–4,13]. The CPS protects bacteria against phagocytosis and impairs host immune activation. Yet, a study reported *S. suis* serotype-dependent differences in bacterial capacity to modulate dendritic cell functions. Authors suggested that differences in CPS composition and charge might differently regulate the interactions with host cells [28]. Knowledge of CPS structure and composition will contribute to better dissect the role of this bacterial component in the pathogenesis of *S. suis* serotype 9 by means of mutagenesis studies

as well as immunological analyses of CPS-mediated modulation of antigen-presenting cells, in comparison to previously described *S. suis* CPSs [29,30].

Acknowledgements

The authors thank Ms. Claudia Duquette for excellent technical assistance during capsular polysaccharide production and purification. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a grant to MS (#342150). GGD is a recipient of a NSERC fellowship.

References

[1] M. Gottschalk, J.J. Zimmerman, L.A. Karriker, A. Ramirez, K.J. Schwartz, G.W. Stevenson (Eds.), In Diseases of Swine (tenth ed.), Wiley-Blackwell: West Sussex, UK (2012), pp. 841-855

[2] N. Charland, J. Harel, M. Kobisch, S. Lacasse, M. Gottschalk. Microbiology, 144 (1998), pp. 325-332

[3] H.E. Smith, M. Damman, J. van der Velde, F. Wagenaar, H.J. Wisselink, N. Stockhofe-Zurwieden, et al. Infect. Immun., 67 (1999), pp. 1750-1756

[4] D. Roy, J.P. Auger, M. Segura, N. Fittipaldi, D. Takamatsu, M. Okura, et al. Can. J. Vet. Res., 79 (2015), pp. 141-146

[5] R. Nomoto, F. Maruyama, S. Ishida, M. Tohya, T. Sekizaki, R. Osawa. Int. J. Syst. Evol. Microbiol., 65 (2015), pp. 438-443

[6] S. Ishida, H.T. Tien le, R. Osawa, M. Tohya, R. Nomoto, Y. Kawamura, et al. J. Microbiol. Methods, 107 (2014), pp. 66-70

[7] H. Zheng, S. Ji, Z. Liu, R. Lan, Y. Huang, X. Bai, et al. Appl. Environ. Microbiol., 81 (2015), pp. 4111-4119

[8] G. Goyette-Desjardins, J.P. Auger, J. Xu, M. Segura, M. Gottschalk. Emerg. Microb. Infect., 3 (2014), p. e45

[9] A. Kerdsin, R. Hatrongjit, M. Gottschalk, D. Takeuchi, S. Hamada, Y. Akeda, et al. J. Microbiol. Immunol. Infect. (2015), 10.1016/j.jmii.2015.1006.1011 (Epub ahead of print)

[10] N. Fittipaldi, M. Segura, D. Grenier, M. Gottschalk. Future Microbiol., 7 (2012), pp. 259-279

[11] M.R. Van Calsteren, F. Gagnon, S. Lacouture, N. Fittipaldi, M. Gottschalk. Biochem. Cell Biol., 88 (2010), pp. 513-525

[12] M.R. Van Calsteren, F. Gagnon, C. Calzas, G. Goyette-Desjardins, M. Okura, D. Takamatsu, et al. Biochem. Cell Biol., 91 (2013), pp. 49-58

[13] M.R. Van Calsteren, G. Goyette-Desjardins, F. Gagnon, M. Okura, D. Takamatsu, R. Roy, et al. J. Biol. Chem., 291 (16) (2016), pp. 8387-8398

[14] N. Charland, J.T. Kellens, F. Caya, M. Gottschalk. J. Clin. Microbiol., 33 (1995), pp. 2220-2221

[15] M. Okura, D. Takamatsu, F. Maruyama, T. Nozawa, I. Nakagawa, M. Osaki, et al. Appl. Environ. Microbiol., 79 (2013), pp. 2796-2806

[16] M. Johnson, I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis, T.L. Madden. Nucleic Acids Res., 36 (2008), pp. W5-W9

[17] T.J. Carver, K.M. Rutherford, M. Berriman, M.A. Rajandream, B.G. Barrell, J. Parkhill. Bioinformatics, 21 (2005), pp. 3422-3423

[18] G.M. Lipkind, A.S. Shashkov, Y.A. Knirel, E.V. Vinogradov, N.K. Kochetkov. Carbohydr. Res., 175 (1988), pp. 59-75

[19] I.C. Schoenhofen, D.J. McNally, E. Vinogradov, D. Whitfield, N.M. Young, S. Dick, et al. J. Biol. Chem., 281 (2006), pp. 723-732

[20] M.S. Kokoulin, A.I. Kalinovsky, N.A. Komandrova, V.E. Tovarchi, S.V. Tomshich, O.I. Nedashkovskaya, et al. Carbohydr. Res., 375 (2013), pp. 105-111

[21] P.E. Jansson, B. Lindberg, U. Lindquist. Carbohydr. Res., 140 (1985), pp. 101-110

[22] S. Park, K.A. Kelley, E. Vinogradov, R. Solinga, C. Weidenmaier, Y. Misawa, et al. J. Bacteriol., 192 (2010), pp. 4618-4626

[23] C. Chen, W. Zhang, H. Zheng, R. Lan, H. Wang, P. Du, et al. J. Clin. Microbiol., 51 (2013), pp. 2582-2591

[24] A. Zhang, M. Yang, P. Hu, J. Wu, B. Chen, Y. Hua, et al. BMC Genom., 12 (2011), p. 523

[25] H.E. Smith, V. Veenbergen, J. van der Velde, M. Damman, H.J. Wisselink, M.A. Smits. J. Clin. Microbiol., 37 (1999), pp. 3146-3152

[26] K. Wang, W. Fan, L. Cai, B. Huang, C. Lu. FEMS Microbiol. Lett., 324 (2011), pp. 117-124

[27] S.D. Bentley, D.M. Aanensen, A. Mavroidi, D. Saunders, E. Rabbinowitsch, M. Collins, et al. PLoS Genet., 2 (2006), p. e31

[28] M. Meijerink, M.L. Ferrando, G. Lammers, N. Taverne, H.E. Smith, J.M. Wells. PLoS One, 7 (2012), p. e35849

[29] C. Calzas, G. Goyette-Desjardins, P. Lemire, F. Gagnon, C. Lachance, M.R. Van Calsteren, et al. Infect. Immun., 81 (2013), pp. 3106-3118

[30] C. Calzas, P. Lemire, G. Auray, V. Gerdts, M. Gottschalk, M. Segura. Infect. Immun., 83 (2015), pp. 441-453

Tables

Table 1. NMR data of *S. suis* serotype 9 capsular polysaccharide[a].

Unit		1	2	3	4	5	6
α-Gal A	H	5.43	3.77	4.00	4.03	4.24	3.73; 3.77
	C	97.2	69.5	70.4	70.4	72.1	62.3
α-Rha B	H	5.02	4.24	3.88	3.51	3.84	1.28
	C	102.1	71.5	80.9	72.1	70.8	17.7
β-Sug C	H	4.83	3.92	4.09		3.65	1.29
	C	103.5	55.5	80.9	93.0	73.6	13.0
β-Gal D	H	4.72	3.95	4.29	4.20	3.80	3.80
	C	103.6	71.6	77.0	68.7	76.1	61.8
Glc-ol E	H	3.68; 3.76	4.00	3.99	3.78	3.83	4.12
	C	63.7	73.4	79.0	70.5	71.2	67.9

[a] Bruker Avance III 600 MHz, at 50 °C. NAc: 2.06/23.8, 176.1 ppm. 31P at 0.4 ppm

Table 2. Genes in the cps locus of *S. suis* serotype 9 encoding enzymes responsible for capsular polysaccharide (CPS) biosynthesis and similarity to genes of other bacteria.

Gene			Similar gene products of *S. pneumoniae*			Similar gene products of *S. saprophyticus* ATCC 15305		
				Amino acids			Amino acids	
	Product (putative)	Function/Pathway	Gene	Identity (%)	Coverage (%)	Locus tag (gene)	Identity (%)	Coverage (%)
cps9A	Integral membrane regulatory protein Wzg	Regulation	*wzg*	57	99	SSP0600	30	39
cps9B	Chain length determinant protein/polysaccharide export protein Wzd	Regulation	*wzd*	61	98	-		
cps9C	Tyrosine-protein kinase Wze	Regulation	*wze*	66	99	SSP0061	35	82
cps9D	Protein-tyrosine phosphatase Wzh	Regulation	*wzh*	62	100	SSP0062	36	81
cps9E	Nucleoside-diphosphate sugar epimerase	4-keto Sug synthesisa	*capD*	49	98	SSP0063 (capD)	51	85
cps9F	Initial sugar transferase (glycosyl-1-phosphate transferase)	Initial transferase #1	*wciI*	62	97	SSP0070	51	92
cps9G	Glycosyltransferase	Transferase #2	*wciV*	40	99	-		

cps9H	Glycosyltransferase	Transferase #3	*wcxS*	31	94	-			
cps9I	LicD-family protein	Transferase #4	*wcrB*	27	86	-			
	Thought to be a glycosyltransferase		*wcyR*	27	91				
cps9J	Oligosaccharide repeat unit polymerase Wzy	Polymerase	-			-			
cps9K	Capsular polysaccharide synthesis protein	Transferase #5	*wcyS*	43	100	-			
cps9L	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	Glucitol synthesisa	*mnp1*	55	97	SSP0354	34	94	
cps9M	NAD-dependent epimerase/dehydratase	Glucitol synthesisa	*mnp2*	46	97	SSP2194	24	72	
cps9N	Flippase Wzx	Transport of repeat units	*wzx*	51	74	-			

[a] Putative functions based on sequence homologies.

Figures

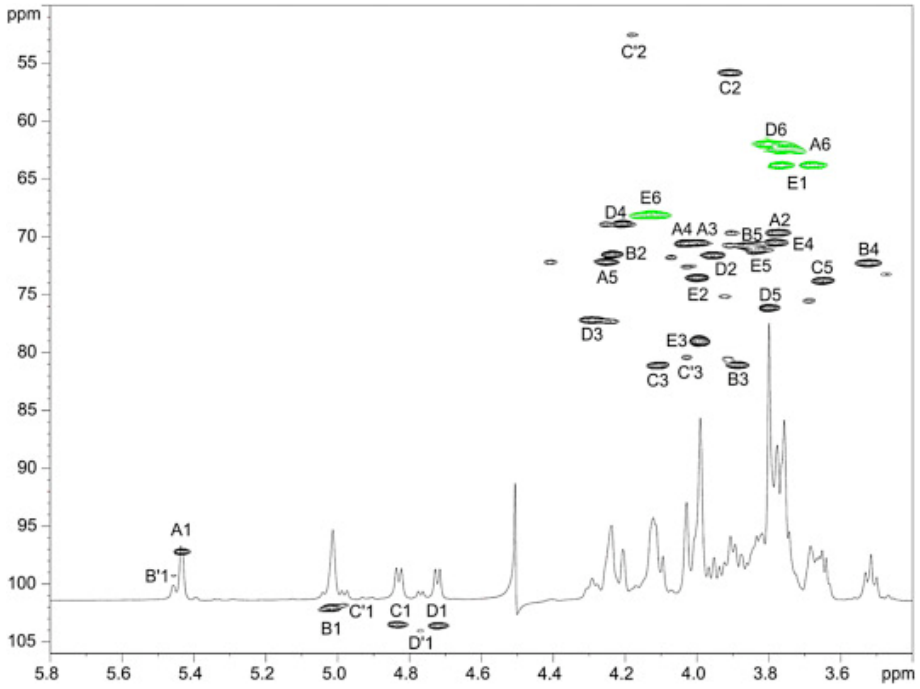


Figure 1. Part of the 1H-13C HSQC and 1H spectra of the *S. suis* serotype 9 capsular polysaccharide (50 °C, 600 MHz).

Minor signals belong to the structures with isomers of Sug C.

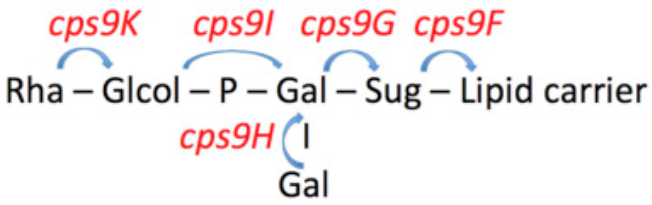


Figure 2. Tentative correlation between structure and genes encoding enzymes responsible for the biosynthesis of *S. suis* serotype 9 capsular polysaccharide (CPS).

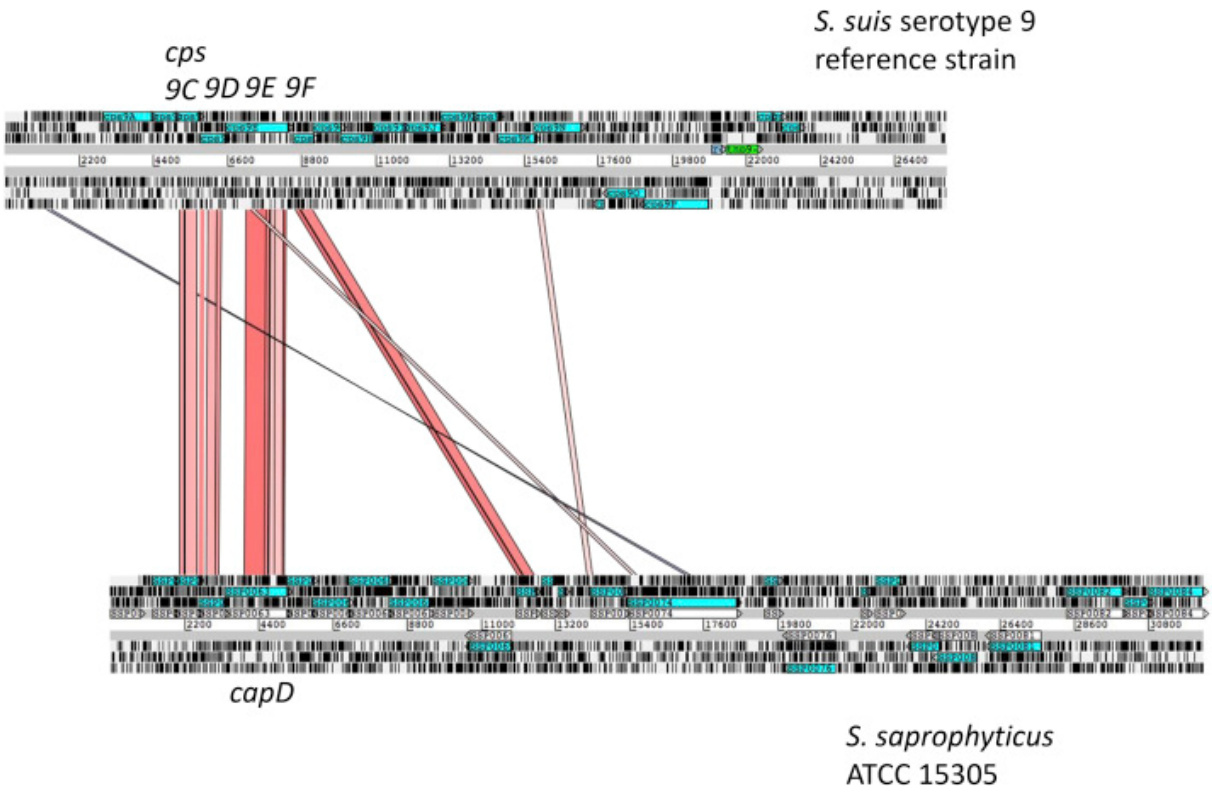


Figure 3. Comparison of capsular polysaccharide (cps) gene clusters between *S. suis* serotype 9 reference strain (GenBank accession no. BR001006) and *Staphylococcus saprophyticus* ATCC 15305 (GenBank accession no. AP008934; nt position, 69784th-102196th).

Tblastx results between the two sequences were displayed by using ACT.

ANNEXES - ARTICLE XII

A single amino acid polymorphism in the glycosyltransferase CpsK defines four *Streptococcus suis* serotypes

---

David Roy [1], Taryn B.T. Athey [2], Jean-Philippe Auger [1], **Guillaume Goyette-Desjardins** [1], Marie-Rose Van Calsteren [1,3], Daisuke Takamatsu [4,5], Masatoshi Okura [4], Sarah Teatero [2], Martín Alcorlo [6], Juan A. Hermoso [6], Mariela Segura [1], Marcelo Gottschalk [1], and Nahuel Fittipaldi [2]

[1] Swine and Poultry Infectious Diseases Research Centre, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC, Canada; [2] Public Health Ontario, Toronto Laboratory, Toronto, ON, Canada;[3] Saint-Hyacinthe Research and Development Centre, Agriculture and Agri-Food Canada, Saint-Hyacinthe, QC, Canada; [4] Division of Bacterial and Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan; [5] The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Gifu, Japan; [6] Department of Crystallography and Structural Biology, Instituto de Química-Física "Rocasolano", Consejo Superior de Investigaciones Científicas, Madrid, Spain.

Publié dans *Scientific Report*. **2017**. 7(1):4066.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé activement à la réalisation des expériences (purifications et analyses physicochimiques; 20%) et à l'analyse des résultats.

Abstract

The capsular polysaccharide (CPS) is the major virulence factor of the emerging zoonotic pathogen *Streptococcus suis*. CPS differences are also the basis for serological differentiation of the species into 29 serotypes. Serotypes 2 and 1/2, which possess identical gene content in their *cps* loci, express CPSs that differ only by substitution of galactose (Gal) by *N*-acetylgalactosamine (GalNAc) in the CPS side chain. The same sugar substitution differentiates the CPS of serotypes 14 and 1, whose *cps* loci are also identical in gene content. Here, using mutagenesis, CPS structural analysis, and protein structure modeling, we report that a single amino acid polymorphism in the glycosyltransferase CpsK defines the enzyme substrate predilection for Gal or GalNAc and therefore determines CPS composition, structure, and strain serotype. We also show that the different CPS structures have similar antiphagocytic properties and that serotype switching has limited impact on the virulence of *S. suis*.

Introduction

Streptococcus suis is a major swine pathogen and an increasingly recognized agent of zoonotic disease [1]. At least 29 *S. suis* serotypes are defined based on a serological reaction directed against the capsular polysaccharide (CPS), a crucial virulence factor with antiphagocytic properties [2, 3, 4, 5]. Strains of serotype 2 are highly prevalent worldwide and frequently isolated from diseased swine [1]. Some serotype 2 genetic lineages such as sequence type (ST) 1, common in European and Asian countries, are highly virulent [1]. Clonal serotype 2 strains belonging to ST7, another highly virulent genotype, were responsible for two major outbreaks of *S. suis* human disease that affected hundreds of patients in China [6]. Other serotype 2 genetic lineages such as ST25 and ST28 are considered less virulent [7], although strains belonging to both ST25 and ST28 have caused human disease [1]. Strains of serotype 14 are also often associated with zoonotic disease [1]. One recurring problem for diagnostics laboratories is that strains of zoonotic serotypes 2 and 14 cross-react in the coagglutination test (the most commonly used *S. suis* serotyping scheme) with strains of non-zoonotic serotypes 1/2 and 1, respectively [8, 9, 10].

CPS biosynthesis in *S. suis* appears to proceed through the flippase/polymerase (Wzx/Wzy)-dependent pathway originally described for lipooligosaccharides biosynthesis [12, 13], in which an initial monosaccharide is linked as a sugar phosphate to a membrane-associated lipid carrier by an initial sugar transferase, followed by sequential addition of sugar residues by specific glycosyltransferases. The repeating units are then translocated across the cytoplasmic membrane by Wzx, polymerized to form the lipid-linked CPS by Wzy, and finally attached to the peptidoglycan by the membrane protein complex [11]. Pioneering work by Smith *et al.* identified that all genes needed for *S. suis* serotype 2 CPS biosynthesis cluster in a single *cps* locus [12, 14]. Further studies identified *cps* loci in all other *S. suis* serotypes [15, 16]. In addition to genes encoding various different glycosyltransferases, polymerases, transferases and translocases, the *cps* loci of some serotypes also contain genes encoding additional enzymes involved in modifications of sugar residues, or in the biosynthesis and linkage of sialic acid to the CPS side chain [12, 14, 15, 16].

We have recently determined the CPS structures of serotypes 2, 1/2, 14 and 1. The serotype 2 CPS contains galactose (Gal), glucose, *N*-acetylglucosamine, rhamnose, and sialic acid [17], while the serotype 14 CPS possesses Gal, glucose, *N*-acetylglucosamine, and sialic acid [18]. The serotype 1/2 CPS differs from the serotype 2 CPS and the serotype 1 CPS from the serotype 14 CPS by a single substitution of the Gal residue bearing the sialic acid in the serotypes 2 and 14 CPS side chains by an *N*-acetylgalactosamine (GalNAc) residue [16, 19] (Fig. 1a-d).

Interestingly, despite the aforementioned differences in CPS sugar composition and structure and the fact that all other serotypes possess a "serotype-specific" gene, serotype pairs 2 and 1/2, and 1 and 14 have identical *cps* gene content (Fig. 1e) [16]. Thus, there is no specific glycosyltransferase permitting to explain the differential addition of Gal or GalNAc to the CPS side chains of these serotypes [16]. To investigate the issue in more detail, we recently sequenced the genomes of seven strains each of serotypes 2 and 1/2, and seven strains each of serotypes 14 and 1. We found that the only consistent difference in the *cps* loci of strains of these serotype pairs was a nonsynonymous single-nucleotide polymorphism (SNP) in codon 161 of gene *cpsK*, predicted to result in a single amino acid difference in the glycosyltransferase CpsK (W161 in serotypes 2 and 14, and C161 in serotypes 1/2 and 1) [20].

Here, we tested the hypothesis that this single amino acid polymorphism is the key factor influencing the sugar residue (Gal or GalNAc) added to the CPS repeating unit by either CpsK variants. We show that polymorphic CpsK variants define expression by *S. suis* strains of either serotype 2 or 1/2 CPSs, or either serotype 14 or 1 CPSs, and that it is possible to achieve serotype switching of field strains of serotypes 2 and 1/2, and 14 and 1 solely by replacing amino acid 161 of CpsK. We also report that serotype switching does not modify the virulence of the strains in an experimental infection model.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table 2. Well-characterized clinical isolates of serotype 2 (strain P1/7) [37], serotype 14 (strain DAN13730) [18], serotype 1/2 (strain 2651) [19] and serotype 1 (strain 1659834) were used. *S. suis* field strains and mutants were grown in Todd-Hewitt broth (THB) or agar (THA) (Becton-Dickinson, Franklin Lakes, NJ, USA) at 37°C. *E. coli* strains were grown in Luria-Bertani broth or agar at 37°C. When needed, antibiotics (Sigma-Aldrich, Oakville, ON, Canada) were added to the culture media at the following concentrations: for *S. suis*, spectinomycin at 100 µg/ml; for *E. coli*, kanamycin and spectinomycin at 50 µg/ml and ampicillin at 100 µg/ml.

DNA manipulations

S. suis genomic DNA was purified using InstaGene Matrix (BioRad, Mississauga, ON, Canada). Oligonucleotide primers (listed in Supplementary Table 3) were from Integrated DNA Technologies (Coralville, IA, USA). Plasmid preparations were performed using the QIAprep Spin Miniprep kit (Qiagen, Toronto, ON, Canada). Restriction enzymes and DNA-modifying enzymes were purchased from ThermoFisher (Waltham, MA, USA) and used according to the manufacturers' recommendations. PCR reactions were carried out with iProof high-fidelity DNA polymerase (BioRad) or with Taq DNA polymerase (Qiagen). Amplification products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced with an ABI 310 automated DNA sequencer using the ABI PRISM dye terminator cycle sequencing kit v3 (ThermoFisher).

Mutant generation

SNP replacements in gene *cpsK* were performed by allelic exchange. PCR amplicons were generated using specific primers and cloned into plasmid pCR2.1 (ThermoFisher), extracted using EcoRI, and subcloned into the thermosensitive *E. coli-S. suis* shuttle vector pSET4s [38] previously digested with EcoRI, giving rise to replacement vectors p4cpskG483T and p4cpskT483G. These vectors were then electroporated into recipient *S. suis* strains using a Biorad Gene Pulser Xcell apparatus (BioRad) under specific conditions (12.5 kV/cm, 200 Ω,

and 25 μF). Isoallelic mutants were isolated as previously described [39]. Sanger sequencing confirmed adequate replacement of nucleotide 483 of *cpsK* genes. Whole-genome sequencing using Illumina MiSeq technology of all parental and mutant strains, and polymorphism identification were performed as previously described [21].

Serotyping

Serotyping was performed by coagglutination as previously described [10]. Results were deemed positive when a strong reaction was obtained within 1 min or less. Dot blot assays were used to confirm the CPS antigenicity of constructed mutants using highly purified CPS preparations, as previously described [19].

Transmission electron microscopy

TEM was carried as previously described [23]. Unless otherwise indicated, chemicals were from Sigma-Aldrich. Briefly, bacteria were grown to mid-logarithmic phase and washed in 0.1 M cacodylate buffer, pH 7.3. Rabbit antisera (150 μl) directed against the different CPS types were used for CPS stabilization. Next, cells were immobilized in 4% (w/v) agar in 0.1 M cacodylate buffer pH 7.3 (Canemco & Marivac, Canton de Gore, QC, Canada). Prefixation was performed adding 0.1 M cacodylate buffer, pH 7.3, containing 0.5% (v/v) glutaraldehyde and 0.15% (w/v) ruthenium red for 30 min. Fixation was performed for 2 h at room temperature with 0.1 M cacodylate buffer, pH 7.3, containing 5% (v/v) glutaraldehyde and 0.05% (w/v) ruthenium red. Post-fixation was carried out with 2% (v/v) osmium tetroxide in water at 4˚C for 16 h. Samples were washed with water every 20 min for 2 h to remove osmium tetroxide and dehydrated in increasing graded series of acetone. Specimens were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin (Electron Microscopy Sciences, Hatfield, PA, USA). Thin sections were post-stained with uranyl acetate and lead citrate and examined with a transmission electron microscope at 80 kV (Hitachi model HT7770, Chiyoda, Tokyo, Japan).

Purification and physicochemical characterization of CPS

S. suis strains were grown in 150 ml of THB at 37°C for 16 h, diluted to 6 l in fresh THB, and grown overnight. The cells were pelleted by centrifugation at 10,000 × *g* for 40 min,

suspended by repeated pipetting in 33 mM phosphate-buffered saline (PBS), pH 8.0, and chilled. The CPSs were then purified as previously described [17]. Purified CPSs were characterized by SEC–MALS, and M_w of each CPS was determined as previously described [18, 40].

NMR spectroscopy

CPSs were exchanged in phosphate buffer, pD 8.0, in D_2O (99.9 atom % D), freeze dried, and dissolved in D_2O (99.96 atom % D) to a final concentration of 33 mM. NMR spectra were acquired on polysaccharide samples at concentrations of 0.4–1.3%. ^1H chemical shifts δ in ppm were referenced to internal deuterated 2,2-dimethyl-2-silapentane-5-sulfonate at δ 0 as recommended by Wishart *et al.* [41]. Spectra were acquired at 11.75 T on a Bruker Avance 500 spectrometer equipped with a 5-mm triple resonance TBI probe with ^1H, ^{13}C, and ^{109}Ag–^{31}P channels at 75–77°C or at 16.45 T on a Bruker Avance 700 spectrometer with a 5-mm cryoprobe with ^1H and ^{13}C channels at 42°C using standard Bruker pulse sequences at the Centre régional de résonance magnétique nucléaire, Department of Chemistry, University of Montreal. Conventional 1D ^1H spectra were acquired with 30° pulses. The gradient-enhanced two-dimensional (ge-2D) COSY spectrum was acquired in magnitude mode using 45° or 90° pulses with or without purge pulses, respectively. Spectra were processed off-line with the software package SpinWorks 4.2.0.0 available at http://home.cc.umanitoba.ca/~wolowiec/spinworks/ For 1D spectra, 32–40 K complex data points were acquired and processed by exponential multiplication with a line-broadening factor equal to the digital resolution, zero filling, complex Fourier transform, phase correction, and fifth-order polynomial baseline correction. Zhu-Bax forward–backward linear prediction with 16 coefficients was systematically applied to 2D processing in the f_1 dimension [42].

Modeling methods

Protein similarity searches were carried out with BLASTP https://blast.ncbi.nlm.nih.gov using the *S. suis* serotype 2 CpsK amino acid sequence against the PDB database. Three available 3D structures showed high identity with the catalytic module of CpsK and were identified as (i) a putative glycosyltransferase from *Streptococcus parasanguinis* (GalT1, PDB code 5hea, identity 39.13% covering 98% sequence), (ii) a

putative glycosyltransferase from *Bacteroides fragilis* (PDB code 3bcv, identity 35.6% covering 88% sequence), and (iii) the chondroitin polymerase from *E. coli* strain K4 in complex with UDP (PDB code 2z87, identity 28% covering 52% sequence). We selected PDB 5hea and used it as a template to build a structural model for *S. suis* CpsK with either W161 or C161. Structural models for both variants were generated independently using the Swiss-Model server [43]. For modeling the interaction with ligands UDP-Gal and UDP-GalNAc, we used the two available 3D structures complexed with substrates that showed the highest identity with *S. suis* CpsK: (i) chondroitin polymerase from *E. coli* strain K4 (K4CP) complexed with UDP-glucuronic acid and UDP (PDB code 2z86 and 2z87, identity 23.22%) and (ii) the human UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferase [44] (pp-GalNAc-T10, PDB code 2D7i, identity 18%). UDP-Gal and UDP-GalNAc PDBs were built using the electronic Ligand Builder eLBOW implemented in Phenix [45]. The generated substrates were structurally superimposed using the UDP-glucuronic acid present in PDB 2z86 as a template.

Experimental animal infection

All experiments involving animals were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and the Use of Laboratory Animals by the Animal Welfare Committee of the University of Montreal, and approved by the latter Committee (Protocol RECH-1570). A well-standardized *S. suis* murine model of infection was used [25]. A total of 80 six-week-old female CD1 mice (Charles River Laboratories, Wilmington, MA, USA) were acclimated to standard laboratory conditions. On the day of the experiment, mice were assigned randomly to 8 groups of 10 mice each. Each group was inoculated by intraperitoneal injection of 1 ml of bacterial suspensions of either one field strain or its derivative mutant expressing a switched serotype. Bacterial inocula were 5×10^7 colony forming units (CFU) for serotypes 2 and 1/2 and corresponding mutants or 1×10^8 CFU for serotypes 14 and 1 and corresponding mutants. These inocula were chosen based on preliminary trials carried out with parental strains and a reduced number of animals (data not shown). Mice were monitored at least three times a day for mortality and clinical signs of systemic disease, such as depression, swollen eyes, rough coat hair, and lethargy. To evaluate bacteremia, blood samples were collected from the tail

vein at 12, 24, 48, and 72 h post-infection, plated onto THA using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA, USA) and bacterial colonies enumerated after incubation at 37°C for 16h.

Phagocytosis assay

Phagocytosis assays were performed using the murine macrophage cell line J774A.1 (ATCC TIB 67) maintained and cultured as previously described [46]. For bacterial phagocytosis, 48 h cell cultures were scraped, washed twice with phosphate-buffered saline (PBS), pH 7.4, and resuspended in antibiotic-free medium at 1×10^5 cells/ml. Cell suspension was then distributed into 24-well tissue culture plates (1 ml/well) and incubated for 3 h to allow cell adhesion. The cell culture medium was removed and cells were infected by adding 250 µl of a 4×10^7 CFU/ml bacterial suspension in culture medium (without antibiotics) and 250 µl of mouse serum (from C56BL/6 mice and stored at −80°C), to obtain a ratio of 100 bacteria per cell. The infected cells were incubated for 60 min at 37°C with 5% CO_2 to allow phagocytosis. Assay conditions were chosen based on preliminary studies (data not shown). After incubation, cells were washed with warm PBS and incubated for 1 h in medium containing 5 µg/ml penicillin G (Sigma-Aldrich) and 100 µg/ml gentamicin (ThermoFisher) to kill extracellular bacteria as previously described [46]. After antibiotic treatment, cells were washed and lysed with 1 ml of sterile distilled water. After vigorous pipetting to ensure complete cell lysis, viable intracellular bacterial counts were determined by plating serial dilutions onto THA using an Autoplate 4000 Automated Spiral Plater. Each test was repeated four times in independent experiments, and the number of CFU recovered per well (mean ± SEM) was determined.

Statistical analysis

All data are expressed as mean ± SEM. *In vitro* data were analyzed for significance using the Student's t-test. Normality was previously verified in order to use Student's t-test. Log-Rank (Mantel-Cox) test was used to analyze survival rates between parental field strains and derivative serotype switching mutants in animal infection assays. Statistical analyses for bacteremia were calculated using the Mann-Whitney Rank Sum test. A P value < 0.05 was used as a threshold for significance.

Results

Exchange of cpsK alleles differing by a single-nucleotide polymorphism between strains of serotypes 2 and 1/2, and between strains of serotypes 14 and 1, results in serotype switching

We hypothesized that the SNP at codon 161 of *cpsK* gene confers CpsK with different substrate predilection and results in the preferential addition of Gal (W161; serotypes 2 and 14) or GalNAc (C161; serotypes 1/2 and 1) residues to the nascent CPS repeating unit. Consequently, replacement of the W161 CpsK variant by the C161 CpsK variant, or vice versa, should result in strain serotype switching. To begin to test this hypothesis, we generated by allelic exchange the following *cpsK* isoallelic mutants: i) strain SS2to1/2 (derived from a serotype 2 field strain, has a W161C substitution in CpsK); ii) strain SS1/2to2 (derived from a serotype 1/2 field strain; has a C161W substitution in CpsK); iii) strain SS14to1 (derived from a serotype 14 field strain; has a W161C substitution in CpsK), and iv) strain SS1to14 (derived from a serotype 1 field strain; has a C161W substitution in CpsK). Whole-genome sequencing of parental and mutant strains confirmed the intended mutation, and did not identify spurious mutations elsewhere in the genome of the mutant strains, with the exception of strain SS1to14, which, compared to the WT serotype 1 strain, presented additional polymorphisms in gene *gatB*, encoding one subunit of a putative aspartyl/glutamyl-tRNA amidotransferase. These additional polymorphisms might impact the pool of arginine and glutamate amino acids of the mutant strain but are unlikely to affect CPS expression.

In all cases, parental and mutant strains expressed CPS of comparable thickness as determined by transmission electron microscopy (TEM) (Supplementary Fig. 1). When examined in the coagglutination test, all mutant strains appeared to have switched serotype (Table 1). However, since the coagglutination test uses polyclonal antibodies that may potentially recognize antigens other than the CPS, we next performed dot blotting with the same antisera and purified CPS from each pair of field and mutant strains. Consistent with the hypothesis of serotype switching, the CPS from the serotype 2 field strain reacted with anti-serotype 2 but not with anti-serotype 1 sera, while the CPS from mutant strain SS2to1/2 (W161C) reacted with both antisera (Fig. 2a, top panel). Essentially similar results were observed for CPS preparations from a serotype 14 field strain and its mutant SS14to1

(W161C) when blotted with anti-serotype 14 and anti-serotype 1 sera (Figure 2b, top panel). As expected, the CPS from the serotype 1/2 field strain reacted with both anti-serotype 1 and anti-serotype 2 sera, while the CPS from mutant strain SS1/2to2 (C161W) reacted with anti-serotype 2 but not with anti-serotype 1 sera (Fig. 2a, bottom panel). Essentially similar results were observed for CPS preparations from the serotype 1 field strain and its mutant SS1to14 (C161W) (Figure 2b, bottom panel), although in this latter mutant, cross-reaction with the anti-serotype 1 serum appeared to be slightly more intense than that observed for the CPS from the field serotype 14 strain. Taken together, these results demonstrate that a single amino acid substitution (W161C or C161W) in the glycosyltransferase CpsK is sufficient to effect serotype switching in each pair of serotypes (2 and 1/2, and 14 and 1).

The W191 CpsK variant adds a Gal residue to the CPS repeating unit; the C191 CpsK variant adds a GalNAc residue instead

To further test the hypothesis that a single amino acid substitution confers CpsK polymorphic variants with different sugar substrate predilection, we next performed nuclear magnetic resonance (NMR) analysis of purified CPSs obtained from each pair of field strains and derivative serotype switching mutants using previously described protocols [17, 18, 19]. The analysis revealed one noticeable additional methyl group signal (δ 2.06) from GalNAc in the one-dimensional (1D) ^{1}H NMR spectrum of the CPS preparation from the SS2to1/2 mutant (Fig. 3a and d), as well as in the spectrum of the CPS preparation of the SS14to1 mutant (Fig 4a and d), compared to CPS preparations from the parental field strains of serotypes 2 (Fig. 3c) and 14 (Fig. 4c). Inversely, this signal was absent from the spectra of CPS preparations from mutants SS1/2to2 (Fig. 3b and f) and SS1to14 (Fig. 4b and f) and present in the spectra of CPS preparations of parental field strains of serotype 1/2 (Fig. 3e) and serotype 1 (Fig. 4e). In the anomeric region, the chemical shift of H-1 of the side-chain 6-substituted residue (GalNAc or Gal) depended on the sugar identity (Fig. 3a and b and Fig. 4a and b).

To determine the position of the H-1–H-2 cross peak, we acquired correlation spectroscopy (COSY) spectra (Supplementary Fig. 2a to d). H-2 resonated at a much higher frequency when an *N*-acetamido moiety instead of a hydroxyl group was present on C-2: for CPSs from SS2to1/2 and SS1/2to2 mutants, the H-1/H-2 signal was found at δ 4.51/3.92 and

4.44/3.54, respectively, as opposed to δ 4.44/3.54 and 4.49/3.94 in the CPSs from parental field strains of serotypes 2 and 1/2, respectively. Similarly, for CPSs from the SS14to1 and SS1to14 mutants, the signal was found at δ 4.52/3.93 and 4.45/3.54, as opposed to δ 4.45/3.54 and 4.51/3.93 in the CPSs from parental field strains of serotypes 14 and 1, respectively [17, 18, 19]. A small shift of the anomeric proton of GlcNAc, to which GalNAc or Gal is attached, was also observed in all cases (Supplementary Fig. 2a to d). Collectively, ^{1}H and COSY NMR spectra unambiguously demonstrated that the SS2to1/2 and the SS1/2to2 mutants synthesized serotypes 1/2 and 2 CPSs, respectively. Similarly, the data unequivocally demonstrated that the SS14to1 and the SS1to14 mutants synthesized serotypes 1 and 14 CPSs, respectively.

It is apparent from the previous results that CPSs expressed by field strains of serotypes 2, 14, 1/2, and 1 (henceforth defined as "native" CPSs) have the same sugar composition and repeating unit structure as those expressed by mutants SS1/2to2, SS1to14, SS2to1/2, and SS14to 1 (henceforth defined as "mutant" CPSs), respectively. To investigate whether other differences existed between native and mutant CPSs, we next performed size-exclusion chromatography coupled with multi-angle light scattering (SEC–MALS). The predicted molecular mass (M_w) of the different CPS preparations were relatively similar between serotype 2 "native" CPS (435 kg/mol) and serotype 2 "mutant" CPS (prepared from strain SS1/2to2) (504 kg/mol), and between serotype 14 "native" CPS (421 kg/mol) and serotype 14 "mutant" CPS (prepared from strain SS1to14 (571 kg/mol), suggesting similar cha**in** lengths. However, "mutant" serotype 1/2 and "mutant" serotype 1 CPSs (prepared from strains SS2to1/2 and SS14to1, respectively) appear to have reduced CPS chain lengths when compared to "native" serotype 1/2 CPS (483 vs. 709 kg/mol) or "native" serotype 1 CPS (490 vs. 741 kg/mol), respectively (Supplementary Table 1). However, it must be noted that data for "mutant" CPS were acquired by analysis of a single batch of CPS preparation per mutant strain. Further experiments are needed to confirm whether those differences in Mw actually represent differences in CPS length.

Three-dimensional modeling of polymorphic CpsK with either W161 or C161 is compatible with substrate predilection for Gal or GalNAc, respectively

Since the only difference between each pair of parental and mutant strains is one SNP in the *cpsK* gene, we concluded from experiments presented above that the polymorphism in amino acid 161 of CpsK is the sole factor determining which sugar residue this glycosyltransferase adds to the CPS repeating unit. To investigate substrate predilection of both polymorphic forms of CpsK in more detail, we next built a three-dimensional (3D) model for the serotype 2 CpsK protein variant (bearing W161) (Fig. 5a). CpsK belongs to the glycosyltransferase family 2 (GT2) that is a member of the clan GT-A, all of which present two tightly associated β/α/β domains that form a central eight-strands β-sheet in a Rossman-like fold. As described in the carbohydrate-active enzymes (CAZY) database (www.cazy.org)[21], GT2 enzymes present an inverting mechanism. Members of this family are responsible, generally, for the transfer of nucleotide-diphosphate sugars to substrates such as polysaccharides and lipids. Strict conservation of the nucleotide-binding site and availability of several 3D structures complexed with different substrates allowed us to identify the localization of the saccharide moieties bound to the activated nucleotide sugar in CpsK. Residue 161 is located at the core of the catalytic center at the beginning of the last β-strand of the central β-sheet and close to the nucleotide-biding site (Fig. 5a). Our modeling analysis revealed that recognition of the nucleotide is not affected by replacement of residues at position 161. Indeed, all the residues required for recognition of the uracil group, the ribose moiety, and the pyrophosphate group are conserved in both variants of CpsK (Fig. 5 and Supplementary Fig. 3).

Docking of uridine diphosphate (UDP)-Gal and UDP-GalNAc in the active sites of both CpsK variants provided a clear explanation of the potential role of residue 161 in substrate specificity. C161 in CpsK from serotype 1/2 could stabilize the GalNAc residue in the UDP-GalNAc substrate by establishing a polar interaction with the acetyl group of the sugar (Fig.5b) (in addition to the potential H-bonds created by the conserved residues with the other oxygen atoms from the GalNAc sugar). The distance between the SH group of C161 and the carbonyl oxygen of GalNAc is in the range of a hydrogen bond formation (3.1-3.7 Å, according to the values stored in the Cambridge Structural Database).

The same happens for the interaction of UDP-Gal with W161-bearing CpsK from serotype 2 (Fig. 5c). In this case, the W161 residue shapes the cavity to accommodate this smaller ligand and provides an H-bond with the O2 of Gal through the N atom of the indole ring (Fig. 5c). Interestingly, this interaction is also observed in the 3D structure of one of CpsK closest homologues, the chondroitin polymerase from *Escherichia coli*, in complex with UDP-glucuronic acid (Protein Data Bank database [PDB] code 2Z86) where one of the N atoms of the side chain of H581 makes an H-bond with the O2 of glucuronic acid (Supplementary Fig. 3b). In both cases, the predicted interactions would contribute to the specificity of each CpsK variant for their respective substrates. However, while each polymorphic form can stabilize its natural substrate (UDP-Gal and UDP-GalNAc in CpsK from serotypes 2 and 1/2, respectively), the exchange of substrates is not possible. With C161, there is enough room to accommodate the *N*-acetyl group bound to the galactosamine moiety at the catalytic groove. However, with W161, the steric hindrance generated between the *N*-acetyl group and the side chain of W161 would prevent the accommodation of UDP-GalNAc at the catalytic groove (Supplementary Fig. 4). Taken together, 3D modeling results provide an explanation to the differential galactosyltransferase and *N*-galactosaminyltransferase activities detected for CpsK W161 and C161 polymorphic variants, respectively.

The CPSs of serotypes 2, 1/2, 14, and 1 have similar antiphagocytic properties, and serotype switching does not alter strain virulence

Although the virulence of *S. suis* is multifactorial, studies with mutant strains impaired in CPS expression have conclusively shown that the CPS plays a key role in the pathogenesis of infection of this pathogen [22, 23, 24]. However, little is known on the effect of serotype switching on the virulence of any given strain. One hypothesis is that inasmuch as the organism expresses a capsule the specific CPS type of any particular strain will not affect virulence. However, the fact that only a few serotypes (notably, serotype 2) predominate among strains isolated from diseased animals and humans (serotype 1/2 strains have never been isolated from human cases so far) might indicate that the specific type of CPS may be important *per se* in defining the virulence of the strain. To begin to differentiate between these hypotheses, we took advantage of the fact that our mutant strains were generated from parental

field strains belonging to different STs with known virulence differences. Indeed, the serotype 2 parental field strain belongs to ST1, the parental serotype 1/2 field strain belongs to ST28, the serotype 14 parental field strain belongs to ST6, and the serotype 1 parental field strain belongs to ST1. Thus, with the exception of the spurious mutation in *gatB* noted above in mutant strain SS1to14, each pair of parental and mutant strains are truly serotype variants that differ by only one SNP genome-wide, and the differences in virulence between pairs of parental field and mutant strains may only result from their different CPSs.

Virulence assay results using a validated murine model of infection [25] showed that the virulence of the SS2to1/2 mutant strain (expressing serotype 1/2 CPS) was virtually identical to that of the parental serotype 2 field strain ($P = 0.4510$). Indeed, both strains caused at least 80% mouse mortality after 3 days post-infection, as expected for highly virulent ST1 strains (Fig. 6a). Mice in both groups had high bacteremia after 24 h post-infection ($P = 0.8917$) (Fig. 6e). In addition, mice infected with either strain showed severe clinical signs such as depression, swollen eyes, rough coat hair, and lethargy. On the other hand, the serotype 1/2 parental strain and its derivative mutant strain SS1/2to2 (expressing serotype 2 CPS) were of low virulence, consistent with previously reported low virulence of ST28 strains [26]. No mortality was recorded in either group ($P = 1.0000$) after 3 days (Fig. 6b), and mice showed only mild clinical signs of infection and low bacteremia in general ($P = 0.1116$) (Fig. 6f). Similarly, no virulence differences were observed between the serotype 14 field strain (ST6 genetic background) and mutant SS14to1 ($P = 0.6273$). Indeed, both groups showed more than 80% mortality, high bacteremia ($P = 0.6842$), and severe clinical signs of infection (Figs. 6c and g). Finally, the virulent serotype 1 ST1 field strain showed high virulence with 70% mortality after 3 days post infection, and mutant SS1to14 induced similar mortality (80%) ($P = 0.6419$) (Fig. 6d). Similarly to other tested ST1 strains, mice infected with either strain showed high bacteremia ($P = 0.5583$) (Fig. 6h) and severe clinical signs.

Next, we investigated *in vitro* the antiphagocytic properties of the different four *S. suis* CPSs by means of phagocytosis assays. Results showed that, independently of CPS type and strain genetic background, all strains were similarly internalized by murine macrophages in the presence (Fig. 7) ($P = 0.9662$ for SS2 and SS2to1/2, $P = 0.8873$ for SS1/2 and SS1/2to2, P

= 0.9874 for SS14 and SS14to1 and *P* = 0.9639 for SS1 and SS1to14) or absence (data not shown) of serum. Thus, substitution of Gal by GalNAc, or *vice versa*, does not significantly alter the antiphagocytic properties of the tested CPSs. Taking the *in vitro* and *in vivo* virulence assays together, we conclude that the different CPSs possess similar antiphagocytic properties, that serotype switching does not impact the virulence of *S. suis* strains that share a similar genetic background, at least for the four serotypes tested here, and that the virulence arsenal particular to the specific genetic background of a given strain is more likely to influence its virulence.

Discussion

Integrated systems biology approaches combining sequencing of multiple genomes of closely related organisms, in combination with animal infection models and relevant *in vitro* approaches, have been instrumental in recognizing the key contribution of small genetic changes such as SNPs and short insertion/deletions to the virulence, phenotypic characteristics, and other important biological traits of strains of several bacterial species [27, 28, 29]. Here, we show that a single amino acid polymorphism in the glycosyltransferase CpsK leads to enzyme variants with differential substrate predilection for Gal and GalNAc, defining the sugar residue added to the CPS repeating unit and thus determining four *S. suis* serotypes. Specifically, we demonstrate that for serotype pairs 2 and 1/2, and 14 and 1, a CpsK variant with W161 results in strains that are serotypes 2 and 14, while a CpsK variant with C161 results in strains that are serotypes 1/2 and 1. Our findings provide a definitive molecular explanation to intriguing previous results showing that strains of serotypes 2 and 1/2 and strains of serotypes 14 and 1 have *cps* loci with identical gene content, but their CPS structures differ between members of each pair, namely by the presence of either a Gal or a GalNAc as the CPS side-chain sugar residue bearing sialic acid [16, 17, 18, 19]. However, NMR is our sole source of structural data, and further studies are needed to elucidate the impact, if any, of the polymorphism on other important CPS characteristics such as the number of synthesized CPS chains and their lengths.

Glycosyltransferases are a large family of proteins that are ubiquitous in bacteria and eukaryotes[30]. Despite the large number of sequence families that have been defined, structural analysis has shown that all but a few glycosyltransferases possess GT-A or GT-B folds. The catalytic domain of the GT-A-fold enzymes can be viewed as a single domain composed by two closely abutting β/α/β Rossmann domains. The Rossmann fold is found in proteins that bind nucleotides and is responsible for binding the nucleotide sugar donor substrate. With only one exception, GT-A enzymes have been found to possess a DXD motif and are metal-ion-dependent glycosyltransferases. The GT-B-fold enzymes possess also two Rossmann domains but separated by a cleft that binds the acceptor. The carboxy-terminal domain is primarily responsible for binding the nucleotide sugar donor substrate. Unlike enzymes that contain the GT-A fold, the GT-B glycosyltransferases are metal-ion independent and do not possess a DXD motif. In this study, the targeted SNP corresponding to amino acid 161 of CpsK protein is located within the glycosyltransferase functional domain. 3D modeling using relevant available crystal structures clearly suggests that the amino acid substitution at position 161 of *S. suis* CpsK leads to conformational and functional changes that permit the enzyme to select between either Gal or GalNAc. A SNP in the gene encoding the glycosyltransferase *wcrL* of *Streptococcus pneumoniae* has been shown to be responsible for the CPS differences observed between serotypes 11A and 11D of that species [31]. However, WcrL variants were shown to have bi-specificity for both Gal and GalNAc, and the resulting CPS differences were due to variable capsular Gal/GalNAc repeat unit ratio[31]. In contrast, our data indicate that *S. suis* CpsK variants are monospecific and incorporate either Gal (W161) or GalNAc (C161).

The CPS plays a key role in *S. suis* virulence. TEM showed that all four isolallelic mutant strains generated here were as encapsulated as their respective parental strains of serotypes 2, 1/2, 14 and 1. Most previous studies have only investigated the impact of abolishing CPS expression on the virulence of the organism [23, 32]. These types of studies cannot differentiate whether a specific CPS composition is important for the virulence of a strain. For example, work on *S. pneumoniae* has shown that specific CPS types endow the strains with differential ability to avoid complement deposition and modulate the virulence of the strain in murine infection models [33, 34]. Previous studies that have compared the virulence of *S. suis* strains expressing different CPS types have, for the most part, used strains with

dissimilar genetic background or whose genetic backgrounds were not known [35]. Here, the use of isoallelic mutants and both in vitro and in vivo infection models permitted us to conclude that the CPS composition plays an unnoticeable role in the virulence of *S. suis* strains of serotypes 2, 1/2, 14, and 1. Indeed, a highly virulent parental ST1 serotype 2 strain was as virulent in mice as its isoallelic mutant expressing serotype 1/2 CPS, while the low virulence of an ST28 serotype 1/2 remained essentially unchanged in its isoallelic mutant expressing type 2 CPS. Similarly, highly virulent ST1 serotype 1 strain expressing serotype 14 CPS and virulent ST6 serotype 14 strain expressing serotype 1 CPS were as virulent as their parental strains. Moreover, we observed no differences in the antiphagocytic properties of CPS 2, 1/2, 14, and 1. One limitation of our study in comparison with the abovementioned work on *S. pneumoniae* is that we evaluated CPS types that differ only by one sugar, i.e., CPS structural changes are relatively minor and may thus not significantly impact virulence. Additionally, it can be hypothesized that the CPSs tested here may possess similar virulence-related properties. Indeed, the cross-reactions between serotypes 2 and 1/2 and serotypes 14 and 1 CPSs in the coagglutination test[16] support the idea that these different CPSs elicit partially overlapping immune responses from the host [19]. *S. suis* strains of serotypes 2 and 14 have caused human disease, while, to our knowledge, strains of serotypes 1 and 1/2 have not [1]. Our results suggest that this differential ability to cause disease in the human host is unlikely to be related to the different compositions and structures of the CPSs of strains of the two serotype pairs.

Small genetic changes such as short insertion/deletions and, particularly, SNPs are key contributors to the genetic diversity of bacterial pathogens [36]. Their impact on bacterial phenotypic traits, including virulence, is only beginning to be uncovered. Here, we show that a single amino acid polymorphism at position 161 of the glycosyltransferase CpsK defines the enzyme specificity for either Gal or GalNAc, and that incorporation of either sugar residue into the CPS repeating unit by polymorphic CpsK is the crucial event in the differentiation between *S. suis* serotypes 2 and 1/2 and between serotypes 14 and 1. Our findings solve a 3-decade long dilemma about the nature of serotyping cross-reactions in *S. suis* serotypes 2, 1/2, 14, and 1 and extend our understanding of how small genetic changes influence bacterial traits and pathogenesis of infection

Acknowledgments

We thank Sonia Lacouture (University of Montreal) for help with *S. suis* collection. We are grateful to Christine Frantz, Aimin Li and staff of Public Health Ontario Genome Core facility for Sanger sequencing of field and mutant strains. We used the NMR processing software SpinWorks, which was developed and is maintained by Kirk Marat, Nuclear Magnetic Resonance Laboratory, Department of Chemistry, University of Manitoba. This work was supported in part by the Natural Sciences and Engineering Research Council of Canada through grants # 04146 to MG and # 342150 to MS, by the Spanish Ministry of Economy and Competitiveness through grant BFU2014-59389-P to JAH, by the biomedicine program of the government of the autonomous community of Madrid through grant S2010/BMD-2457 to JAH, and by Public Heath Ontario through internal grant PIF 2014-005 to NF. DR is the recipient of a Fonds de recherche du Québec-Nature et technologies (FRQ-NT) Doctoral Scholarship and has also received a scholarship from the Swine and Poultry Infectious Disease Research Centre.

References

1. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. *Emerg Microb Infect* **3**, e45 (2014).

2. Baums CG, Valentin-Weigand P. Surface-associated and secreted factors of *Streptococcus suis* in epidemiology, pathogenesis and vaccine development. *Anim Health Res Rev* **10**, 65-83 (2009).

3. Gottschalk M, Segura M. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. *Vet Microbiol* **76**, 259-272 (2000).

4. Hill JE, Gottschalk M, Brousseau R, Harel J, Hemmingsen SM, Goh SH. Biochemical analysis, *cpn60* and 16S rDNA sequence data indicate that *Streptococcus suis* serotypes 32 and 34, isolated from pigs, are *Streptococcus orisratti*. *Vet Microbiol* **107**, 63-69 (2005).

5. Tien le HT, Nishibori T, Nishitani Y, Nomoto R, Osawa R. Reappraisal of the taxonomy of *Streptococcus suis* serotypes 20, 22, 26, and 33 based on DNA-DNA homology and *sodA* and *recN* phylogenies. *Vet Microbiol* **162**, 842-849 (2013).

6. Yu H*, et al.* Human *Streptococcus suis* outbreak, Sichuan, China. *Emerg Infect Dis* **12**, 914-920 (2006).

7. Fittipaldi N*, et al.* Lineage and virulence of *Streptococcus suis* serotype 2 isolates from North America. *Emerg Infect Dis* **17**, 2239-2244 (2011).

8. Higgins R, Gottschalk M. An update on *Streptococcus suis* identification. *J Vet Diagn Invest* **2**, 249-252 (1990).

9. Perch B, Kjems E, Slot P, Pedersen KB. Biochemical and serological properties of R, S, and RS streptococci. *Acta Path Micro IM B* **89**, 167-171 (1981).

10. Gottschalk M, Higgins R, Jacques M, Mittal KR, Henrichsen J. Description of 14 new capsular types of *Streptococcus suis*. *J Clin Microbiol* **27**, 2633-2636 (1989).

11. Yother J. Capsules of *Streptococcus pneumoniae* and other bacteria: paradigms for polysaccharide biosynthesis and regulation. *Annu Rev Microbiol* **65**, 563-581 (2011).

12. Smith HE, de Vries R, van't Slot R, Smits MA. The *cps* locus of *Streptococcus suis* serotype 2: genetic determinant for the synthesis of sialic acid. *Microb Pathog* **29**, 127-134 (2000).

13. Lairson LL, Henrissat B, Davies GJ, Withers SG. Glycosyltransferases: structures, functions, and mechanisms. *Annu Rev Biochem* **77**, 521-555 (2008).

14. Smith HE*, et al.* Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. *Infect Immun* **67**, 1750-1756 (1999).

15. Wang K, Fan W, Cai L, Huang B, Lu C. Genetic analysis of the capsular polysaccharide synthesis locus in 15 *Streptococcus suis* serotypes. *FEMS Mimcrobiol Lett* **324**, 117-124 (2011).

16. Okura M*, et al.* Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: potential mechanisms for generation of capsular variation. *Appl Environ Microbiol* **79**, 2796-2806 (2013).

17. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. *Biochem Cell Biol* **88**, 513-525 (2010).

18. Van Calsteren MR*, et al.* Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide. *Biochem Cell Biol* **91**, 49-58 (2013).

19. Van Calsteren MR*, et al.* Explaining the serological characteristics of *Streptococcus suis* serotypes 1 and 1/2 from their capsular polysaccharide structure and biosynthesis. *J. Biol Chem* **291**:8387-98 (2016).

20. Athey TB, Teatero S, Lacouture S, Takamatsu D, Gottschalk M, Fittipaldi N. Determining *Streptococcus suis* serotype from short-read whole-genome sequencing data. *BMC Microbiol* **16**, 162 (2016).

21. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res* **42**, D490-495 (2014).

22. Houde M, Gottschalk M, Gagnon F, Van Calsteren MR, Segura M. *Streptococcus suis* capsular polysaccharide inhibits phagocytosis through destabilization of lipid microdomains and prevents lactosylceramide-dependent recognition. *Infect Immun* **80**, 506-517 (2012).

23. Roy D*, et al.* Role of the capsular polysaccharide as a virulence factor for *Streptococcus suis* serotype 14. *Can J Vet Res* **79**, 141-146 (2015).

24. Chabot-Roy G, Willson P, Segura M, Lacouture S, Gottschalk M. Phagocytosis and killing of *Streptococcus suis* by porcine neutrophils. *Microb Pathog* **41**, 21-32 (2006).

25. Dominguez-Punaro MC, Segura M, Plante MM, Lacouture S, Rivest S, Gottschalk M. *Streptococcus suis* serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection. *J Immunol* **179**, 1842-1854 (2007).

26. Auger JP, Fittipaldi N, Benoit-Biancamano MO, Segura M, Gottschalk M. Virulence studies of different sequence types and geographical origins of *Streptococcus suis* serotype 2 in a mouse model of infection. *Pathogens* **5**, pii: E48 (2016).

27. Katz LS*, et al.* Using single-nucleotide polymorphisms to discriminate disease-associated from carried genomes of *Neisseria meningitidis*. *J Bacteriol* **193**, 3633-3641 (2011).

28. Laabei M*, et al.* Predicting the virulence of MRSA from its genome sequence. *Genome Res* **24**, 839-849 (2014).

29. Read TD, Massey RC. Characterizing the genetic basis of bacterial phenotypes using genome-wide association studies: a new direction for bacteriology. *Genome Med* **6**, 109 (2014).

30. Kapitonov D, Yu RK. Conserved domains of glycosyltransferases. *Glycobiology* **9**, 961-978 (1999).

31. Oliver MB*, et al. Streptococcus pneumoniae* serotype 11D has a bispecific glycosyltransferase and expresses two different capsular polysaccharide repeating units. *J Biol Chem* **288**, 21945-21954 (2013).

32. Feng Y*, et al.* Attenuation of *Streptococcus suis* virulence by the alteration of bacterial surface architecture. *Sci Rep* **2**, 710 (2012).

33. Melin M, Trzcinski K, Meri S, Kayhty H, Vakevainen M. The capsular serotype of *Streptococcus pneumoniae* is more important than the genetic background for resistance to complement. *Infec Immun* **78**, 5262-5270 (2010).

34. Kelly T, Dillard JP, Yother J. Effect of genetic switching of capsular type on virulence of *Streptococcus pneumoniae*. *Infect Immun* **62**, 1813-1819 (1994).

35. Meijerink M, Ferrando ML, Lammers G, Taverne N, Smith HE, Wells JM. Immunomodulatory effects of *Streptococcus suis* capsule type on human dendritic cell responses, phagocytosis and intracellular survival. *PloS one* **7**, e35849 (2012).

36. Pearson T, Okinaka RT, Foster JT, Keim P. Phylogenetic understanding of clonal populations in an era of whole genome sequencing. *Infect Genet Evol* **9**, 1010-1019 (2009).

37. Holden MT*, et al.* Rapid evolution of virulence and drug resistance in the emerging zoonotic pathogen *Streptococcus suis*. *PLoS One* **4**, e6072 (2009).

38. Takamatsu D, Osaki M, Sekizaki T. Thermosensitive suicide vectors for gene replacement in *Streptococcus suis*. *Plasmid* **46**, 140-148 (2001).

39. Roy D, Grenier D, Segura M, Mathieu-Denoncourt A, Gottschalk M. Recruitment of factor H to the *Streptococcus suis* cell surface is multifactorial. *Pathogens* **5**, (2016).

40. Calzas C*, et al.* Group B *Streptococcus* and *Streptococcus suis* capsular polysaccharides induce chemokine production by dendritic cells via Toll-like receptor 2- and MyD88-dependent and -independent pathways. *Infect Immun* **81**, 3106-3118 (2013).

41. Wishart DS*, et al.* 1H, 13C and 15N chemical shift referencing in biomolecular NMR. *J Biomol NMR* **6**, 135-140 (1995).

42. Zhu GB, Bax A. Improved linear prediction of damped NMR signals using modified "Forward-Backward" linear prediction. *J Magn Reson* **100**, 202-207 (1992).

43. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res* **31**, 3381-3385 (2003).

44. Kubota T*, et al.* Structural basis of carbohydrate transfer activity by human UDP-GalNAc: polypeptide alpha-N-acetylgalactosaminyltransferase (pp-GalNAc-T10). *J Mol Biol* **359**, 708-727 (2006).

45. Adams PD*, et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Cryst D* **66**, 213-221 (2010).

46. Segura M, Stankova J, Gottschalk M. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. *Infect Immun* **67**, 4646-4654 (1999).

Tables

Table 1. Results of the coagglutination test.

Strain	Tested antisera			Interpretation
	Anti-serotype 1	Anti-serotype 2	Anti-serotype 14	
SS2	-	+		Serotype 2
SS2to1/2	+	+		Serotype 1/2
SS1/2	+	+		Serotype 1/2
SS1/2to2	-	+		Serotype 2
SS14	-		+	Serotype 14
SS14to1	+		+	Serotype 1
SS1	+		+	Serotype 1
SS1to14	-		+	Serotype 14

Figures

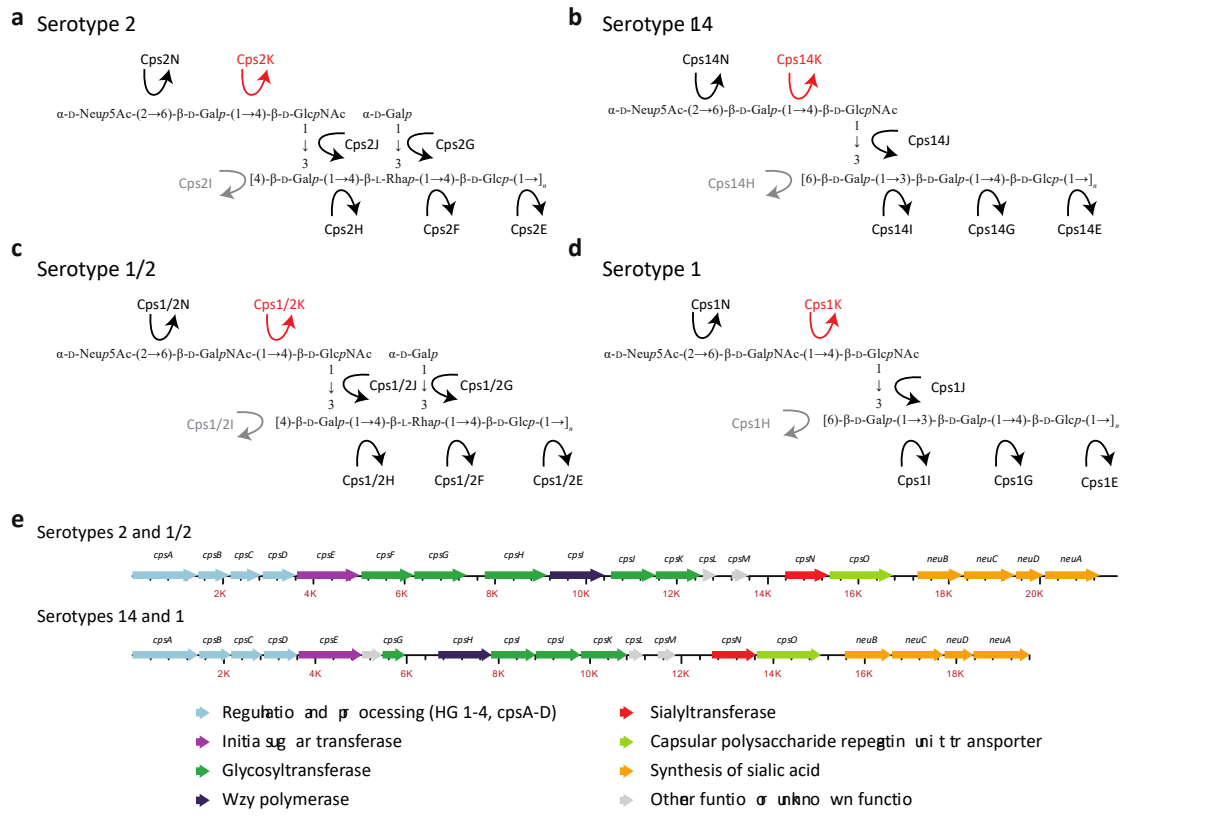


Figure 1. Capsular polysaccharide structures of *S. suis* serotypes 2, 1/2, 14, and 1 capsular polysaccharides, and schematics of the *cps* loci of these serotypes.

a–d, CPS structures of serotype 2 (a), serotype 14 (b), serotype 1/2 (c), and serotype 1 (d). In serotypes 1 and 1/2, CpsK is predicted to catalyze the transfer of the side chain *N*-acetylgalactosamine (GalNAc) residue to the CPS repeating unit, while in serotypes 2 and 14, CpsK would catalyze the transfer of the galactose (Gal) residue at the same corresponding side chain. (e), All enzymes involved in CPS biosynthesis, including CpsK, are encoded by genes located in a single *cps* locus. Serotypes 2 and 1/2 and serotypes 14 and 1 have identical CPS gene content and organization. The putative functions of the enzymes encoded by *cps* genes are depicted with different colors. Please note that Cps enzymes (and *cps* genes) have been renamed compared to previous publications [12, 14, 16, 17, 18, 19] to reflect recent developments in actual or predicted function.

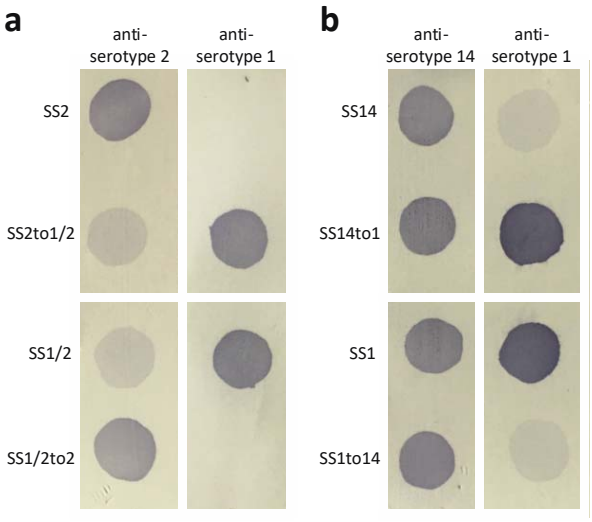


Figure 2. Serotype switching of mutants is confirmed by dot blotting of purified CPS preparations and specific antisera.

a, the CPS from a serotype 2 field strain reacts with anti-serotype 2 but not with anti-serotype 1 sera, while the CPS from mutant strain SS2to1/2 (W161C) reacts with both antisera (top panel). The CPS from a serotype 1/2 field strain reacts with both anti-serotype 1 and anti-serotype 2 sera, while the CPS from mutant strain SS1/2to2 (C161W) reacts with anti-serotype 2 but not with anti-serotype 1 sera (bottom panel). b, the CPS from a serotype 14 field strain reacts strongly with anti-serotype 14 and weakly with anti-serotype 1 sera, while the CPS from mutant strain SS14to1 (W161C) reacts strongly with both antisera (top panel). The CPS from a serotype 1 field strain reacts strongly with both anti-serotype 14 and anti-serotype 1 sera, while the CPS from mutant strain SS1to14 (C161W) reacts strongly with anti-serotype 14 but weakly with anti-serotype 1 sera (bottom panel).

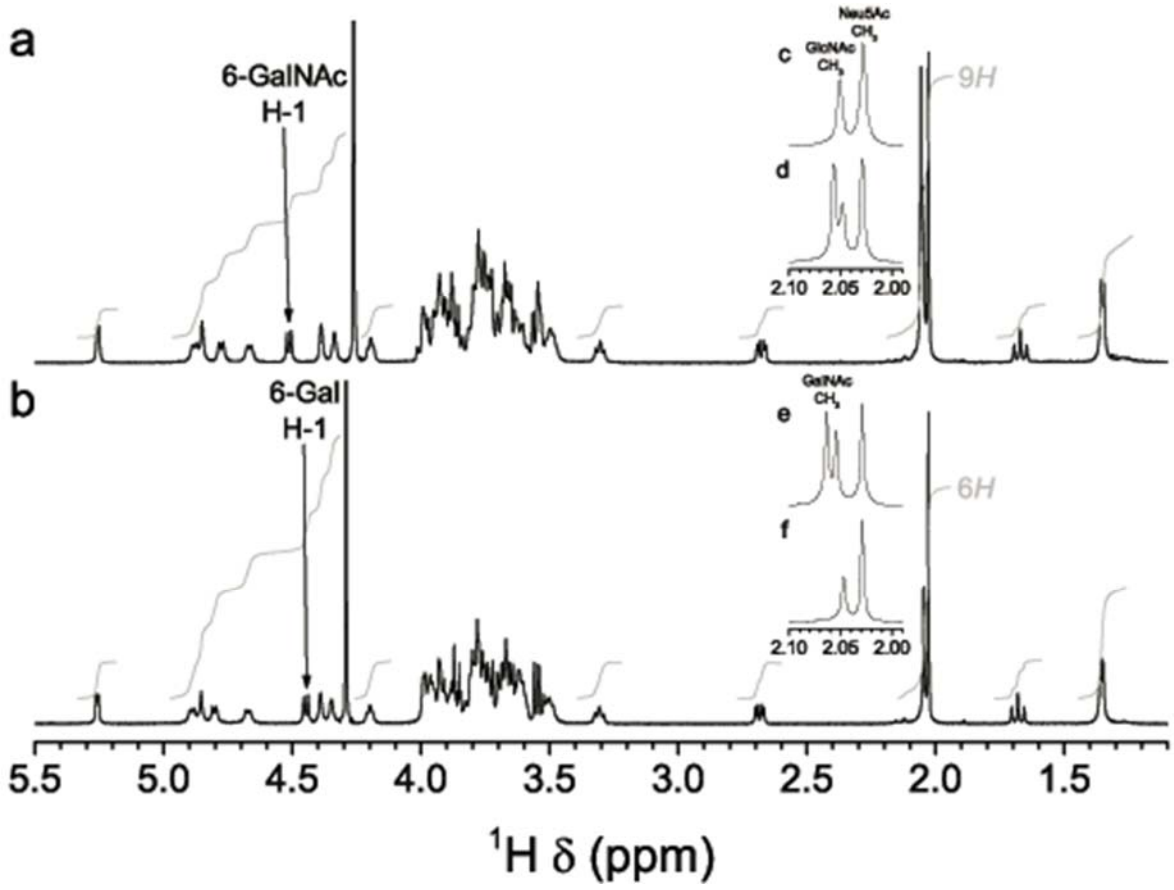


Figure 3. 1D ^{1}H NMR spectra of CPS preparations from serotypes 2 and 1/2 in 33 mM phosphate pD 8.0 in D_2O.

a–b, Full spectrum. c–f, Expansion of the methyl region. a, d, SS2to1/2 mutant, 500 MHz, 77°C. b, f, SS1/2to2 mutant, 500 MHz, 75°C. c, Serotype 2 field strain, 600 MHz, 50°C [17]. e, Serotype 1/2 field strain, 700 MHz, 42°C [19]. Specific characteristics of the ^{1}H NMR spectrum due to the presence of GalNAc in the native serotype 1/2 CPS were also found in the spectrum of the CPS from the corresponding mutant expressing serotype 1/2 CPS. Conversely, the spectrum of the CPS from the mutant expressing serotype 2 CPS, as well as that of the native serotype 2 CPS, lacked the signal attributed to *N*-acetyl of GalNAc.

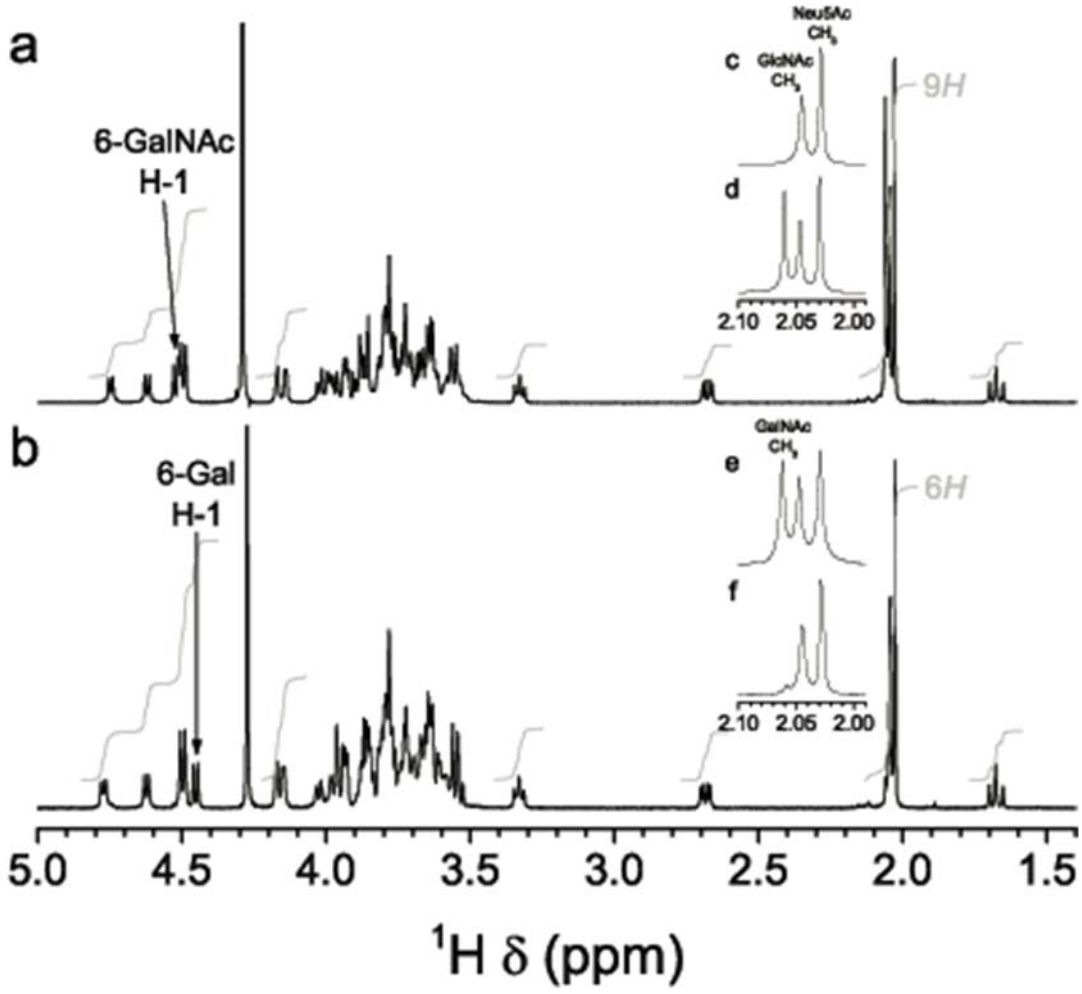


Figure 4. 1D ^{1}H NMR spectra of CPS preparations from serotypes 14 and 1 in 33 mM phosphate pD 8.0 in D_2O.

a–b, Full spectrum. c–f, Expansion of the methyl region. a, d, SS14to1 mutant, 500 MHz, 75°C. b, f, SS1to14 mutant, 500 MHz, 77°C. c, Serotype 14 field strain, 500 MHz, 77°C [18]. e, Serotype 1 field strain, 700 MHz, 70°C [19]. Specific characteristics of the ^{1}H NMR spectrum due to the presence of GalNAc in the native serotype 1 CPS were also found in the spectrum of the CPS from the corresponding mutant expressing serotype 1 CPS. Conversely, the spectrum of the CPS from the mutant expressing serotype 14 CPS, as well as that of the native serotype 14 CPS, lacked the signal attributed to *N*-acetyl of GalNAc.

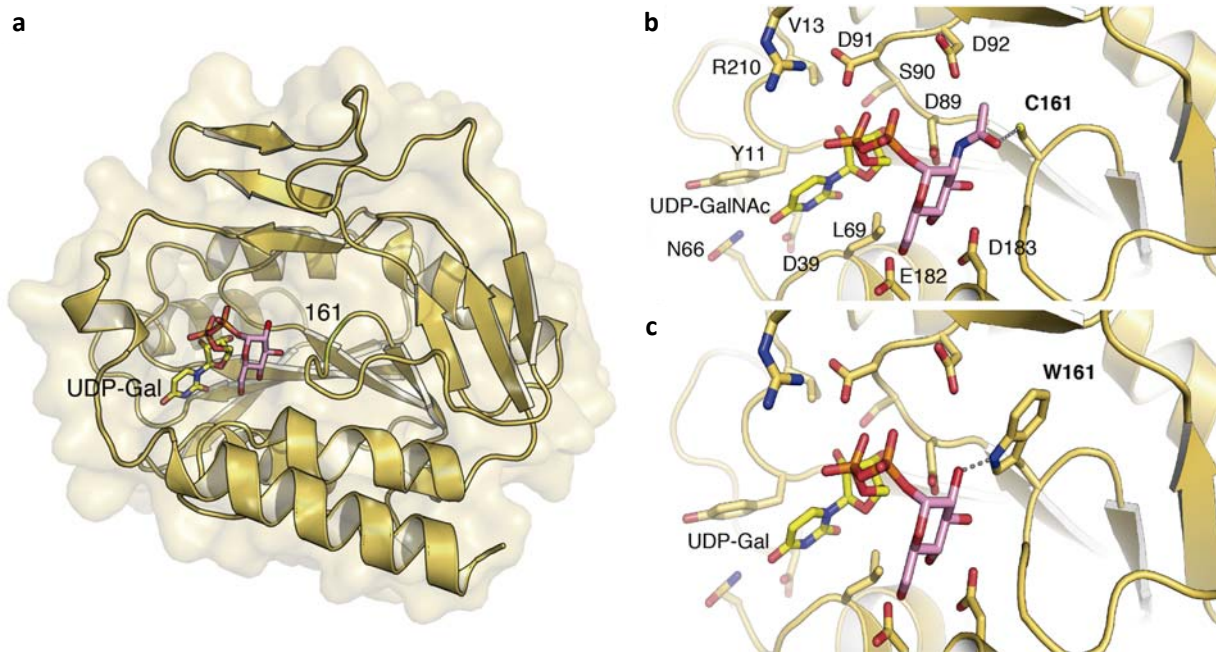


Figure 5. Three-Dimensional modeling of CpsK polymorphic variants.

a, The serotype 2 CpsK protein structure is depicted in yellow ribbon, and the position of amino acid residue 161 was colored in green and labeled. The docked substrate UDP-Gal is shown in sticks with the Gal moiety in pink. b, Detailed view of the catalytic center of protein CpsK from serotype 1/2 with a cysteine residue at position 161 (C161) in complex with UDP-GalNAc as a substrate. Residues predicted to play a role in substrate binding and stabilization are depicted as capped sticks and labeled. Potential hydrogen bond between C161 and *N*-acetyl group of GalNAc is represented with a dashed grey line. c, Same view as in panel a for the catalytic center of protein CpsK from serotype 2 with a tryptophan residue at position 161 (W161) in complex with UDP-Gal molecule as a substrate. Dashed grey line represents the potential hydrogen bond between W161 and the hydroxyl group of Gal.

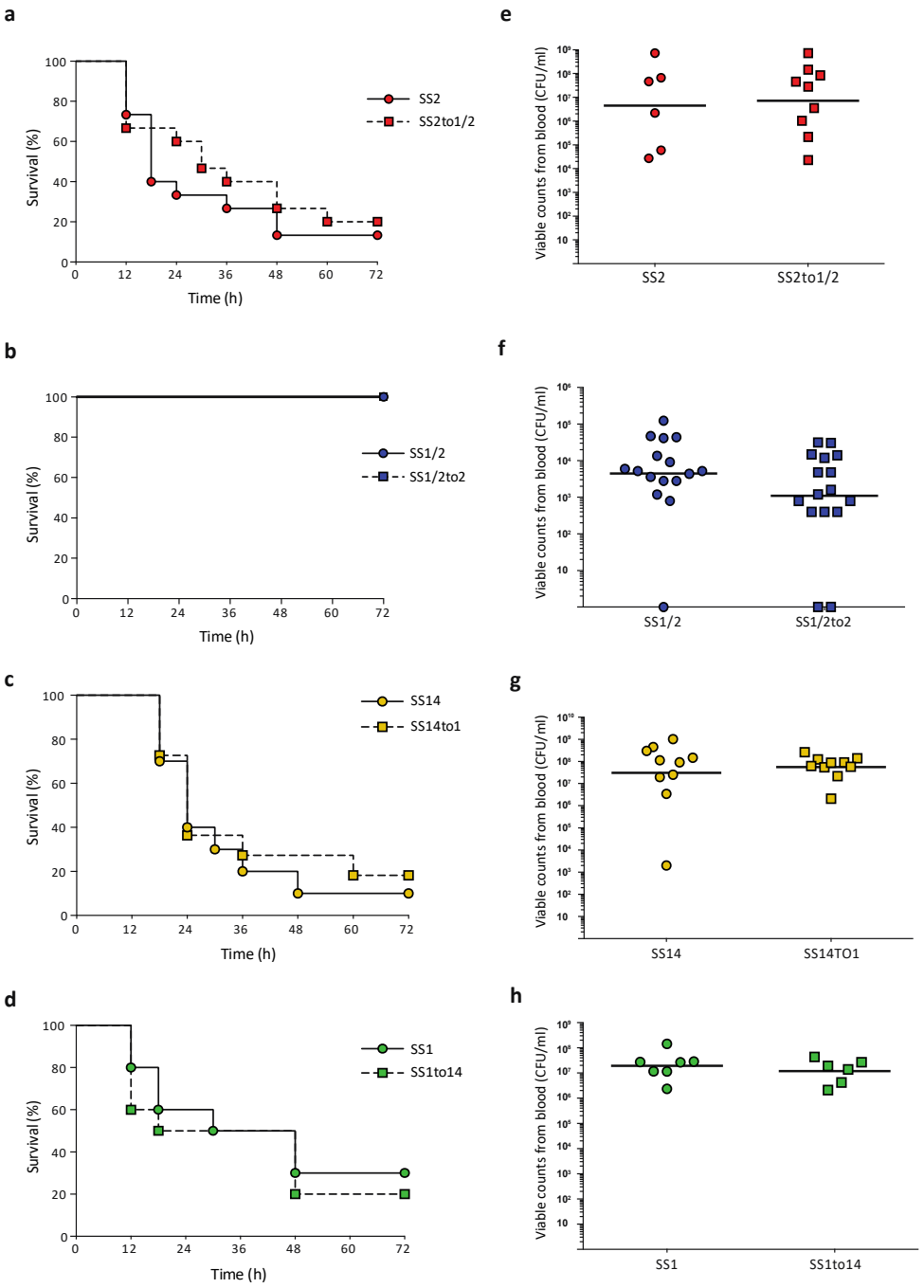


Figure 6. Serotype switching does not impact strain virulence.

a–d, Survival of CD1 mice inoculated by intraperitoneal injection with either 5×10^7 CFU of serotype 2 (a) or 1/2 (b) strains or with either 1×10^8 CFU of serotype 14 (c) or 1 (d) strains. In all cases, Log-Rank (Mantel-Cox) test revealed no significant differences in survival rates between the field parental strains and derivative mutants. All control animals injected with

vehicle (Todd Hewitt Broth) survived the trial (data not shown for simplicity). e–h, Bacterial load in blood was evaluated in all groups by drawing 5 µl of blood from the tail vein of mice followed by plating and enumeration (see methods). e, Serotype 2 field strain and derived mutant SS2to1/2. f, Serotype 1/2 field strain and derived mutant SS1/2to2. g, Serotype 14 field strain and derived mutant SS14to1. h, Serotype 1 field strain and derived mutant SS1to14. No significant differences in bacterial load were observed between parental strains and their corresponding mutants (Mann-Whitney Rank Sum test, $P<0.05$).

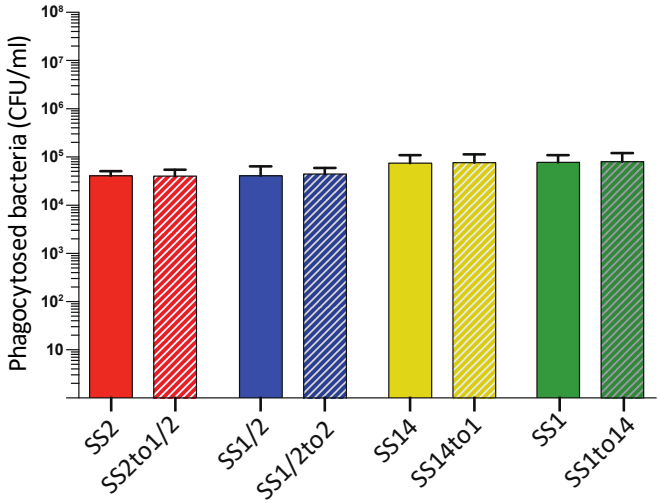


Figure 7. The antiphagocytic properties afforded to *S. suis* by CPS types 2, 1/2, 14, and 1 appear similar under *in vitro* conditions.

Parental strains and isogenic mutants (1×10^7 CFU/ml) were incubated for 60 min with J774 macrophages (multiplicity of infection = 100) in the presence of 50% murine serum. Results represent the mean (CFU/ml) + SEM of four independent experiments. Statistical analyses using the Student's t-test showed no significant differences in the number of internalized bacteria between strains.

Supporting information

Supplementary material is available with the article through the journal Web site at https://www.nature.com/articles/s41598-017-04403-3#Sec20.

Supplementary Table I. Average yields for CPS purification of the different *S. suis* field and mutant strains and CPS molecular weights.

Supplementary Table II. Bacterial strains and plasmids used in this study.

Supplementary Table III. Oligonucleotide primers used in this study.

Supplementary Figure 1. Transmission electron micrographs.

Supplementary Figure 2. Portion of the 500 MHz ge 2D NMR COSY spectrum of *S. suis* CPSs.

Supplementary Figure 3. Structural and substrate-binding conservation in closest homologues of CpsK from *S. suis*.

Supplementary Figure 4. Steric hindrance between tryptophan 161 and the N-acetyl group of UDP-GalNAc.

ANNEXES - ARTICLE XIII

Capsular Sialyltransferase Specificity Mediates Different Phenotypes in *Streptococcus suis* and Group B *Streptococcus*

David Roy [1], Daisuke Takamatsu [2,3], Masatoshi Okura [2], **Guillaume Goyette-Desjardins** [1], Marie-Rose Van Calsteren [1,4], Audrey Dumesnil [1], Marcelo Gottschalk [1], and Mariela Segura [1]

[1] Swine and Poultry Infectious Disease Research Centre, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC, Canada

[2] Division of Bacterial and Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Kannondai, Tsukuba, Ibaraki, Japan

[3] The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Gifu, Japan

[4] Saint-Hyacinthe Research and Development Centre, Agriculture and Agri-Food Canada, Saint-Hyacinthe, QC, Canada

Publié dans *Frontiers in Microbiology*. **2018**. 9:545.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé activement à la réalisation des expériences (purifications et analyses physicochimiques; 20%) et à l'analyse des résultats.

Abstract

The capsular polysaccharide (CPS) represents a key virulence factor for most encapsulated streptococci. *Streptococcus suis* and Group B *Streptococcus* (GBS) are both well-encapsulated pathogens of clinical importance in veterinary and/or human medicine and responsible for invasive systemic diseases. *S. suis* and GBS are the only Gram-positive bacteria which express a sialylated CPS at their surface. An important difference between these two sialylated CPSs is the linkage between the side-chain terminal galactose and sialic acid, being α-2,6 for *S. suis* but α-2,3 for GBS. It is still unclear how sialic acid may affect CPS production and, consequently, the pathogenesis of the disease caused by these two bacterial pathogens. Here, we investigated the role of sialic acid and the putative effect of sialic acid linkage modification in CPS synthesis using inter-species allelic exchange mutagenesis. To this aim, a new molecular biogenetic approach to express CPS with modified sialic acid linkage was developed. We showed that sialic acid (and its α-2,6 linkage) is crucial for *S. suis* CPS synthesis, whereas for GBS, CPS synthesis may occur in presence of an α-2,6 sialyltransferase or in absence of sialic acid moiety. To evaluate the effect of the CPS composition/structure on sialyltransferase activity, two distinct capsular serotypes within each bacterial species were compared (*S. suis* serotypes 2 and 14 and GBS serotypes III and V). It was demonstrated that the observed differences in sialyltransferase activity and specificity between *S. suis* and GBS were serotype unrestricted. This is the first time that a study investigates the interspecies exchange of capsular sialyltransferase genes in Gram-positive bacteria. The obtained mutants represent novel tools that could be used to further investigate the immuno-modulatory properties of sialylated CPSs. Finally, in spite of common CPS structural characteristics and similarities in the *cps* loci, sialic acid exerts differential control of CPS expression by *S. suis* and GBS.

1. Introduction

Capsular polysaccharides (CPSs) play critical roles in the pathogenesis of the disease caused by several bacterial pathogens, including streptococci. Indeed, the CPS expressed at the bacterial surface is one of the primary structures that interacts with host cells during colonization - the first step of the infection – and, more importantly, during invasion and dissemination within the host. *Streptococcus suis* and Group B *Streptococcus* (GBS; also known as *Streptococcus agalactiae*) are two well-encapsulated Gram-positive bacteria that were extensively studied in the last years/decades due to their veterinary and/or medical significance. Variations in CPS antigenicity allow these two bacterial species classification into serotypes, which differ in their clinical importance and epidemiological features, including geographical distribution (1-3).

Streptococcus suis is a zoonotic pathogen that causes severe economic problems in swine production and represents a serious risk for public health. The most common clinical outcomes caused by *S. suis* in pigs are meningitis and septicemia with sudden death. In humans, meningitis and severe streptococcal toxic shock-like syndrome is also frequently reported, especially in Asian countries. Other pathologies include arthritis, endocarditis, and pneumonia. Of the initially described 35 capsular types or serotypes, *S. suis* type 2 predominates worldwide in both pigs and humans. Besides this important and highly virulent serotype, type 14 is also emerging as a threat to human health (2). On the other hand, GBS is an important cause of severe invasive bacterial infections in humans worldwide (3, 4). Clinical manifestations of GBS infection include pneumonia, septicemia, and meningitis in newborns and infants. GBS diseases also occur in pregnant women and have been recognized as an emerging cause of life-threatening invasive infections in adults, particularly the elderly and immunocompromised patients. To date, GBS is classified into ten different serotypes, and type III is the most common type in GBS meningitis, whereas serotype V has long been recognized as a leading cause of invasive disease in adults (3, 4).

Besides the common feature of being encapsulated and inducing similar pathologies, *S. suis* and GBS both use the Wzx/Wzy-dependent pathway to express their CPSs (1, 5). The Wzx/Wzy-dependent pathway is characterized by the implication of two key enzymes; the

Wzy polymerase and the Wzx flippase. The CPS structures of GBS types III and V (6-8) and of *S. suis* types 2 and 14 (9, 10) have already been determined. Although *S. suis* types 2 and 14 and GBS types III and V share common CPS structural elements, the CPS plays different roles in the pathogenesis of the disease. Indeed, it was demonstrated that the *S. suis* CPS is a critical antiphagocytic factor that protects bacteria against phagocytosis by macrophages, dendritic cells and neutrophils (11-14). The *S. suis* CPS is thus considered as a shielding factor that allows bacterial evasion of immunoclearance and characterizes *S. suis* as a strictly extracellular pathogen. In contrast, GBS is easily internalized at high numbers by dendritic cells and macrophages in spite of a thick CPS, being able to survive intracellularly for a transient period of time (15-17).

Another striking feature of these two pathogens is that *S. suis* and GBS are the only Gram-positive bacteria expressing a sialylated CPS. Interestingly, there is a difference in the linkage between the side-chain terminal galactose and sialic acid. Indeed, *S. suis* expresses sialic acid α-2,6-linked to the adjacent galactose rather than an α-2,3-linked sialic acid as is the case for GBS (5, 9, 10) (Figure 1). It was hypothesized that the type of sialic acid linkage may differently modulate immune cell activation and, consequently, may have an impact on bacterial-host interactions (18). Yet, this remains to be investigated in the context of *S. suis* and GBS infections.

In this study, we firstly evaluated the role of sialic acid in the synthesis and export of CPS by Gram-positive bacteria by deletion of genes encoding the sialyltransferases or those involved in the sialic acid synthesis pathway. Secondly, to specifically study the role of sialic acid linkage in *S. suis* (α-2,6) and GBS (α-2,3), we constructed *S. suis* type 2 or type 14 substitution mutants possessing the GBS type III α-2,3-sialyltransferase instead of the native α-2,6-sialyltransferase. Conversely, we constructed GBS type III and type V mutants possessing the exogenous *S. suis* α-2,6-sialyltransferase. Using this novel genetic approach, we demonstrated a critical role of not only the presence of sialic acid, but more importantly its type of linkage in *S. suis* CPS production by two distinct serotypes. In contrast, GBS was still able to express asialo CPS or α-2,6-linked sialylated CPS irrespectively of the serotype. This

is the first time that a study investigates the interspecies exchange of capsular sialyltransferase genes in Gram-positive bacteria.

2. Material and Methods

2.1. Plasmids, bacterial strains, and culture conditions

The well-encapsulated virulent *S. suis* serotype 2 strain P1/7, *S. suis* serotype 14 strain DAN13730, GBS type III strain COH1, and GBS type V strain CJB111 (ATCC BAA-23) were used as the host (wild-type) strains for in-frame allelic deletion mutagenesis. Bacterial strains and plasmids used in this study are listed and described in Table 1. Streptococcal strains were grown in Todd-Hewitt broth (THB) or agar (THA) (Becton-Dickinson, Sparks, MD) at 37°C. *Escherichia coli* strains were grown in Luria-Bertani broth or agar (Becton-Dickinson) at 37°C. When needed, antibiotics (Sigma, Oakville, ON, Canada) were added to the culture media at the following concentrations: for *S. suis*, spectinomycin (Sp) at 100 µg/ml; for *E. coli*, kanamycin and Sp at 50 µg/ml, and ampicillin at 100 µg/ml. Experiments were carried out in a BSL-2 certified laboratory.

2.2. DNA manipulations

S. suis genomic DNA was purified by InstaGene Matrix solution (BioRad Laboratories, Hercules, CA). Accession numbers for reference *cps* loci sequences are: *S. suis* serotype 2: BR001000; *S. suis* serotype 14: AB737822; GBS type III: HG939456.1; and GBS type V: NC_004116. Transformations of *E. coli* were performed as recommended by the manufacturer (Invitrogen, Burlington, ON, Canada). Extraction and purification of recombinant plasmids were performed with QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). Restriction enzymes were purchased from TaKaRa Bio (Otsu, Shiga, Japan) and used according to the manufacturer's directions. Alkaline phosphatase for plasmid dephosphorylation was purchased from MP Biomedicals (Solon, OH). PCR reactions were carried out with the iProof proofreading DNA polymerase (BioRad Laboratories) or with Taq DNA polymerase (Qiagen). Oligonucleotide primers were from IDT (Coralville, IA) and are listed in **Supplementary Table S1**. Amplification products were purified with the QIAgen PCR purification kit (Qiagen) and sequenced with an ABI 310 automated DNA sequencer

using the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

2.3. RT-PCR analysis of the S. suis cps locus

Bacterial RNA from serotype 2 strain P1/7 overnight culture was extracted with TRIZOL reagent (Invitrogen) as recommended by the manufacturer. RNase-free DNase (BioRad Laboratories) was used to treat RNA samples to remove contaminant genomic DNA. Reverse-transcriptase experiments were carried out with Qiagen One Step RT-PCR kit following the manufacturer's protocol. Primers used for RT-PCR experiments (Supplementary Table S1) were designed for each gene within the *cps* locus based on the available *S. suis* serotype 2 *cps* locus sequence (Accession # BR001000). PCR amplification from cDNA was carried out with NEB Taq polymerase (NEB, Ipswich, MA) with the following specific cycling conditions: 94°C for 5 min followed by 40 cycles at 94°C for 30 s, 47°C for 30 s, and 72°C for 1 min/kb. RNA samples without reverse-transcription step were used as templates to verify absence of genomic DNA in the samples.

2.4. Indirect deletion of the sialyltransferase gene (cps2N) in S. suis serotype 2

In order to inhibit CPS production and thus by-pass the lethality induced by mutation of the sialyltransferase (*cps2N*) gene in *S. suis* (19), we developed a 'three-step' approach based on gene deletion/insertion by double cross-over homologous recombination to delete the *cps2N* sialyltransferase gene in *S. suis* (Figure 2).

First, precise in-frame deletion in the *neu2C* gene was achieved using splicing-by-overlap-extension PCR (20) as previously described, to generate the SS2Δsynth (Δ*neu2C*) non-encapsulated mutant. The *neuC* mutation was previously shown to be non-lethal and was already successfully complemented (21, 22). Briefly, overlapping PCR products generated by PCR were cloned into the pCR2.1 TA-cloning vector (Invitrogen), extracted using restriction-enzyme digestion, and cloned into the thermosensitive *E. coli-S. suis* shuttle vector pSET4s, giving rise to the p4Δ*neuC* mutation vector. Final constructions of the pSET4s vector were electroporated into *S. suis* competent cells with a Biorad Gene Pulser Xcell apparatus (BioRad Laboratories) under specific conditions (12.5 kV/cm, 200 Ω, and 25 □F), and cells were

plated on THA supplemented with Sp (THA+SP) and incubated for 3 days at 28°C. Several Sp-resistant colonies were then subcultured again on THA+SP for 3 days at 28°C. Next, the candidates were cultured on THA+SP and incubated at 37°C for two successive passages and then screened for first crossing-over event. Loss of vector was induced by incubation of candidates at 28°C. Temperature and Sp-resistant clones were successively cultured on THA and THA+SP to obtain Sp-sensitive candidates. Deletion of the gene was confirmed by PCR and sequence analysis.

Next, to knockdown *cps2N* and obtain the double mutant SS2Δsynth/ΔsiaT *(*Δ*neu2C*/Δ*cps2N*),* the plasmid p4Δ*cps2N* was also constructed by using splicing-by-overlap-extension PCR as described above (20). The p4Δ*cps2N* plasmid was then introduced into the competent SS2Δsynth strain. Electroporation and SS2Δsynth/ΔsiaT (SS2Δ*neu2C*/Δ*cps2N*) double mutant construction were carried out under the same aforementioned conditions (23).

Finally, in order to reintroduce a functional *neuC* gene into the double mutant SS2Δsynth/ΔsiaT *(*Δ*neu2C*/Δ*cps2N*), the intact *neuC* gene with corresponding upstream/downstream regions of *neuC* was amplified by PCR from P1/7 strain and cloned into the pSET4s vector as described above, giving rise to p4*neuC*. The p4*neuC* plasmid was then introduced into the competent double mutant SS2Δsynth/ΔsiaT *(*Δ*neu2C*/Δ*cps2N*) to obtain the indirect deletion mutant SS2ΔsiaT (Δ*cps2N*). Electroporation and mutant construction were carried out as already described (23). Deletion/insertion of targeted genes was confirmed by PCR and sequence analysis. Moreover, giving the fact that mutations in the *cps* locus (mostly in *cps2E* and *cps2F*) can occur during such deletion (19), we also sequenced *cps2E* and *cps2F* genes in SS2ΔsiaT mutant and confirmed that no mutation was present.

2.5. Indirect substitution of the sialyltransferase gene (cpsN) in S. suis serotypes 2 and 14

To obtain sialyltransferase substitution mutants in *S. suis* serotypes 2 and 14, we used the same approach as for the indirect deletion of *cps2N*. Precise in-frame deletion in *neu2C* (serotype 2) and *neu14C* (serotype 14) genes (UDP-*N*-acetylglucosamine 2-epimerase; sialic acid synthesis) was achieved using the p4Δ*neuC* mutation vector as described above. The same vector (p4Δ*neuC*) was used for both serotypes 2 and 14, as corresponding sequences

were 100% identical. Again, this step was required in order to inhibit CPS production and thus by-pass the lethality induced by mutation of the sialyltransferase gene in *S. suis* (19).

Gene substitution plasmids p4sia2,3_2 and p4sia2,3_14 were constructed by overlapping PCR, merging the intact *cps3K* gene (coding for the GBS α-2,3-sialyltransferase) with upstream and downstream coding regions of *cps2N* and *cps14N* genes (coding for the *S. suis* α-2,6-sialyltransferases). Overlapping PCR products generated by PCR were then cloned into the thermosensitive pSET4s vector. Resulting substitution plasmids p4sia2,3_2 and p4sia2,3_14 were introduced into competent *S. suis* SS2Δsynth (serotype 2) and SS14Δsynth (serotype 14) mutants under the same aforementioned electroporation conditions. Substitution mutants SS2Δ*neu2C*/*cps3K* (SS2Δsynth/sia2,3) and SS14Δ*neu14C*/*cps3K* (SS14Δsynth/sia2,3) were obtained as described for other *S. suis* mutants (23).

Finally, in order to reintroduce a functional *neuC* gene into SS2Δ*neu2C*/*cps3K* and SS14Δ*neu14C*/*cps3K* mutants, the p4*neuC* plasmid was introduced into competent SS2Δ*neu2C*/*cps3K* and SS14Δ*neu14C*/*cps3K* mutants to obtain indirect substitution mutants SS2sia2,3 and SS14sia2,3. The same vector (p4*neuC*) was used for both serotypes 2 and 14, as corresponding sequences were 100% identical. Electroporation and mutant construction were carried out as described above. Gene substitution was confirmed by PCR and sequence analysis. As for SS2ΔsiaT mutant, we also sequenced *cpsE* and *cpsF* genes in SS2Δ*neu2C*/*cps3K* and SS14Δ*neu14C*/*cps3K* mutants and confirmed absence of mutations.

2.6. Construction of the complemented Δcps2N mutant (SS2compΔsiaT)

The intact *cps2N* gene was amplified from genomic DNA of *S. suis* serotype 2 wild-type strain with primers containing specific restriction sites (**Supplementary Table S1**). PCR products and pMX1 vectors were then digested with the appropriate restriction enzyme before ligation. Final constructions were cloned into *E. coli* MC1061. The plasmid pMX1 is a derivative of the S. suis–E. coli shuttle cloning vector pSET2 and possesses the S. suis malQ promoter for transgene expression in S. suis (24). Complementation of the SS2ΔsiaT (Δcps2N) mutant was achieved by transformation with the pMX1*cps2N* complementation vector by electroporation under the same aforementioned conditions. Presence of the plasmid within the

complemented mutant was confirmed by PCR. Plasmid stability was evaluated by measuring bacterial growth rates under selective pressure (Sp; 100 µg/ml). As for SS2ΔsiaT mutant, we sequenced *cps2E* and *cps2F* genes to confirm that no mutations occurred during transformation procedures.

2.7. Deletion mutants in GBS type V

Precise in-frame deletion in *cps5K* (GBSVΔsiaT) and *neu5B* (GBSVΔsynth) genes was achieved using splicing-by-overlap-extension PCR (20). Overlapping PCR products generated by PCR were cloned into the plasmid pCR2.1 (Invitrogen), extracted using restriction-enzyme digestion, and cloned into the thermosensitive *E. coli-S. suis* shuttle vector pSET4s, giving rise to the p4Δ*cps5K* and p4Δ*neu5B* mutation vectors. Final constructions of the pSET4s vector (p4Δ*cps5K* and p4Δ*neu5B*) were electroporated into GBS type V competent cells. Electroporation and mutant construction were carried out as described previously for *S. suis* (23). Deletions of the *cps5K* and *neu5B* genes in GBSVΔsiaT and GBSVΔsynth, respectively, were confirmed by PCR and sequence analysis.

2.8. Exogenous sialyltransferase exchange in GBS types III and V

In contrast to *S. suis*, in order to substitute GBS type III and V sialyltransferases, direct gene replacement by double-crossover homologous recombination system was used. The upstream region and downstream region of GBS sialyltransferase *cps3K* or *cps5K* genes (accession numbers: AAD53072 and NP_688172, respectively) were amplified by PCR, conserving the intact stop codon of the upstream coding gene and the intact start codon of the downstream coding gene. In addition, the complete intact *cps2N* sialyltransferase gene (accession number: CAR45180) of *S. suis* serotype 2 strain P1/7 was amplified by PCR. The PCR products were then merged together by overlapping PCRs. The cloning step in pCR2.1 and pSET4s was done as described above, giving rise to substitution vectors p4sia2,6_III and p4sia2,6_V. Final constructions (p4sia2,6_III and p4sia2,6_V) were introduced into competent GBS under the same electroporation conditions than for *S. suis*. Substitution mutants GBSIIIsia2,6 and GBSVsia2,6 were obtained as described for GBS deletion mutants. Gene substitution was confirmed by PCR and sequence analysis.

2.9. Hydrophobicity test

In order to have a qualitative first impression of CPS expression by different mutant strains, *S. suis* and GBS mutants were tested (triplicate independent assays) for cell surface hydrophobicity by measuring their absorption to *n*-hexadecane according to the procedure previously described (25). Reference strains of *S. suis* serotype 2 (P1/7), *S. suis* serotype 14 (DAN13730), GBS serotype III (COH1), and GBS serotype V (CJB111) were used as positive controls. Non-encapsulated mutants SS2Δ*cps*, SS14Δ*cps,* GBSIIIΔ*cps*, and GBSVΔ*cps* were used as reference for a non-encapsulated phenotype. Since the hydrophobicity test has limited sensitivity, other methods were also used to confirm CPS expression (see points 2.11 and 2.12-14).

2.10. Whole-bacterial cell enzyme-linked lectin assay (ELLA)

In order to investigate the specific linkage of sialic acid in the sialyltransferase substitution mutants, a whole-bacterial cell ELLA was carried out with the biotinylated *Sambucus nigra* agglutinin (SNA-I, Vector Labs Canada, Burlington, ON, Canada) and the biotinylated *Maackia amurensis* leukoagglutinin (MAL-I, Vector Labs) which specifically recognize sialic acid as Neu5Acα-2,6-Gal*p*/Gal*p*NAc or as Neu5Acα-2,3-Galβ-1,4-GlcNAc, respectively (26, 27). The test was based on a previously described technique and adapted for whole bacteria (28). A 10-ml overnight culture in THB inoculated with the appropriate strains was harvested by centrifugation, washed and resuspended in 10 ml of PBS. The suspension was then diluted 10X in PBS (10^7 CFU/ml), and 100 □l was distributed into wells of an ELISA plate (Nunc-Immuno Polysorp, Canadawide Scientific, Toronto, ON, Canada). Wells were then dried overnight, fixed with 50 µl of 100% high-quality methanol and dried for 20 min. After coating, the wells were washed and blocked by the addition of Carbo-Free solution 1X (Vector Labs). After washing, the wells were incubated 1 h with biotinylated SNA-I or biotinylated MAL-I followed by horseradish peroxidase-labeled Avidin D (Vector Labs), and 3,3',5,5'-tetramethylbenzidine was finally added for detection. The enzyme reaction was stopped with the addition of 1 N H_2SO_4, and the absorbance was read at 450 nm with an ELISA plate reader.

2.11. Transmission electron microscopy (TEM)

TEM was carried out to confirm CPS expression at the bacterial surface of different mutant strains as previously described (22). Briefly, bacteria were grown to mid-logarithmic phase and resuspended in 0.1 M cacodylate buffer pH 7.3 containing 2.5% (v/v) glutaraldehyde and 0.05% (w/v) ruthenium red. Fixation was performed for 2 h at room temperature. Ferritin (Electron Microscopy Sciences, Hatfield, PA) was then added to a final concentration of 1 mg/ml and incubated for 30 min at room temperature. Afterwards, cells were immobilized in 4% (w/v) agar in 0.1 M cacodylate buffer pH 7.3 and post-fixed with 2% (v/v) osmium tetroxide in water overnight at 4°C. Samples were washed with water every 20 min for 2 h to remove osmium tetroxide and dehydrated in an increasing graded series of acetone. Specimens were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin (Electron Microscopy Sciences). Thin sections were post-stained with uranyl acetate and lead citrate and examined with a transmission electron microscope at 80 kV (model JEM 1230, Jeol, Tokyo, Japan).

2.12. CPS purification from GBS mutants

CPSs from the GBS type III mutant GBSIIIsia2,6 and GBS type V mutants GBSVsia2,6, GBSVΔsynth, and GBSVΔsiaT were purified as previously described (29). Briefly, 8 l of THB (Oxoid, Thermo Fisher Scientific, ON, Canada) was inoculated with an overnight culture of the appropriate strain (1:40 dilution) and incubated until OD_{540} reached 0.8. The bacterial cells were harvested by centrifugation at 10,000 *g* for 40 min, washed in PBS pH 7.3 and treated with 1 N NaOH at 37°C overnight. After neutralization and dialysis, proteins were digested with 1 mg/ml pronase (Sigma) at 37°C overnight. After subsequent dialysis, the CPSs were treated with 0.8 M acetic anhydride (Sigma) in 5 N NaOH for re-*N*-acetylation of polysaccharides. The CPSs were finally purified by gel filtration on Sephacryl S-300 (GE Healthcare, Little Chalfont, UK), using 50 mM NH_4HCO_3 as the eluent. Control native CPSs were also purified from respective wild-type strains.

2.13. Nuclear magnetic resonance (NMR) spectroscopy

Purified CPSs from GBSVΔsynth, GBSVΔsiaT, GBSVsia2,6, and GBSIIIsia2,6 mutants were exchanged in phosphate buffer pD 8.0 in D_2O (99.9 atom % D), freeze dried,

and dissolved in D_2O (99.96 atom % D) to a final concentration of 33 mM. The other polysaccharides were exchanged in D_2O (99.9 atom % D), freeze dried, and dissolved in D_2O (99.96 atom % D). NMR spectra were acquired on polysaccharide samples at concentrations of 0.1–1.1%. ^1H chemical shifts δ in ppm were referenced to internal deuterated 2,2-dimethyl-2-silapentane-5-sulfonate (DSS-d_6) at δ 0 as recommended by (30). A Chemagnetics (Fort Collins, CO) CMX Infinity 300 spectrometer was used for 7.05-T experiments with a 5-mm dual $^{13}C/^1H$ Nalorac probe (Martinez, CA) at 60°C. The one-dimensional (1D) ^1H experiment was performed with the original pulse program of the Spinsight software. The 16 K complex data points were acquired and processed by exponential multiplication with a line broadening factor equal to the digital resolution, complex Fourier transform, phase correction, and fifth-order polynomial baseline correction. Alternatively, spectra were acquired at 11.75 T on a Bruker Avance 500 spectrometer equipped with a 5-mm triple resonance TBI probe with ^1H, ^{13}C, and $^{109}Ag–^{31}P$ channels at 60–80°C or at 16.45 T on a Bruker Avance 700 spectrometer with a 5-mm cryoprobe with ^1H and ^{13}C channels at 65°C using standard Bruker pulse sequences at the Centre régional de résonance magnétique nucléaire (Department of Chemistry, University of Montreal). Conventional 1D ^1H spectra were acquired with 90° or 30° pulses with or without solvent presaturation. The *z*-restored spin-echo was used to acquire 1D ^1H-decoupled ^{13}C spectrum of straight baseline. The 1D distortionless enhancement by polarization transfer (DEPT) spectrum with adiabatic pulses was recorded with a reading pulse of 135° and the free-precession period optimized for 145-Hz one-bond coupling constant. The gradient-enhanced two-dimensional (ge-2D) correlation spectroscopy (COSY) spectrum was acquired in magnitude mode. The phase-sensitive 2D total correlation spectroscopy (TOCSY) spectrum with Malcom Levitt's sequence (MLEV) was acquired with or without presaturation and an effective spin lock time of 80 ms. The phase-sensitive 2D rotating-frame nuclear Overhauser spectroscopy (ROESY) spectrum with presaturation or using purging pulses was acquired with a mixing time of 300 ms. The phase-sensitive ge-2D heteronuclear single-quantum coherence (HSQC) experiment using echo–antiecho and adiabatic pulses for inversion and refocusing and Bloch-Siegert effects was optimized for 145–155 Hz. The phase-sensitive ge-2D HSQC–TOCSY experiment with MLEV using echo–antiecho was performed with a delay optimized for a 140–145-Hz coupling constant and a mixing time of 80 ms. The phase-sensitive ge-2D

heteronuclear multiple-bond correlation (HMBC) experiment using a three-fold low-pass *J*-filter was run without ^{13}C decoupling with one-bond and long-range delays optimized for 145 and 1–8 Hz, respectively. Bruker spectra were processed off-line with SpinWorks (Copyright, Kirk Marat, University of Manitoba [http://home.cc.umanitoba.ca/~wolowiec/spinworks/]). For 1D spectra, 29–64 K complex data points were acquired and processed by exponential multiplication with a line broadening factor equal to the digital resolution, zero filling, complex Fourier transform, phase correction, and fifth-order polynomial baseline correction. Zhu-Bax forward–backward linear prediction with 16 coefficients was systematically applied to 2D processing in the f_1 dimension (31).

2.14. Weight-average molecular mass characterization of purified CPSs

The weight-average molecular mass (M_w) of each CPS was determined by size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) as described by (29). The chromatographic separation was performed with two 8 mm x 300 mm Shodex OHpak gel filtration columns connected in series (SB-806 and SB-804), preceded by an SB-807G guard column (Showa Denko, Tokyo, Japan). Elution was done with a Waters 510 pump (Waters, Milford, MA), using a 0.1 M $NaNO_3$ mobile phase filtered through a 0.02-µm membrane (Whatman, Maidstone, UK), at a flow rate of 0.5 ml/min. Samples were dissolved in the SEC eluent at concentrations of 0.7–1.0 mg/ml and then injected with a 100 or 200 µl sample loop. Molecular masses were determined with a Dawn EOS MALS detector (Wyatt, Santa Barbara, CA). A model RI 410 differential refractometer (Waters) was used as a concentration detector. A refractive index increment (dn/dc) of 0.137 ml/g was calculated for 690 nm, using data for xanthan at 436 and 546 nm, and the second virial coefficient (A_2) was taken as zero. Calculations were performed with the ASTRA software, version 6.0.0.108 (Wyatt).

2.15. Statistical analysis

All data are expressed as mean ± SEM and were analyzed for significance using the Student *t*-test. Normality was previously verified in order to select the appropriate test. A P value < 0.05 was used as a threshold for significance.

3. Results

3.1. Transcription of the S. suis cps locus occurs in a single polycistronic transcript

To confirm that the *cps* locus of *S. suis* is encoding a single polycistronic transcript and to confirm that the sialyltransferase is under the same promoter than other CPS synthesis genes, RNA obtained from an overnight culture of *S. suis* was analyzed by RT-PCR. Using appropriate primers from adjacent genes (Supplementary Table S1), we showed that all genes within the *cps* coding locus are transcripted as a single polycistronic transcript (**Figure 3**). Indeed, RT-PCR products for all primers within the *cps* locus were obtained. Negative RT-PCR amplifications were obtained with reactions R1 and R23, delimiting the mRNA transcript. Genomic DNA was used to confirm primer efficiency for R1 and R23 as shown with R2 and R24, respectively. No amplified products were present in negative-control reaction (R25) without RT. These results confirmed that the *cps* locus of *S. suis* is encoding a single polycistronic transcript under the regulation of the same promoter (Figure 3).

3.2. Deletion of the sialyltransferase in S. suis results in a non-encapsulated phenotype

It has been previously reported that deletion of the *neuC* gene (UDP-*N*-acetylglucosamine 2-epimerase; sialic acid synthesis) in *S. suis* serotypes 2 and 14 results in a non-encapsulated phenotype (Table 1). However, the phenotypic outcome of a deletion of the sialyltransferase (*cpsN*) gene has never been addressed. Given the fact that mutations blocking side-chain assembly (*cpsJ*), polymerization (*cpsL*), sialylation (*cpsN*), or exportation (*cpsO*) are lethal for *S. suis* (19), we developed a three-step mutagenesis approach in order to by-pass the lethality of sialyltransferase (*cpsN*) mutation. It has been shown that mutation in the sialyltransferase can occur naturally in *S. suis* in presence of a suppressive mutation in other CPS synthesis genes that results in CPS inhibition (19). We thus took advantage of this particularity in order to knockout the sialyltransferase gene in *S. suis* serotype 2 (mutant SS2ΔsiaT; see experimental procedures). As depicted in Figure 4A, the hydrophobicity of the SS2ΔsiaT mutant was very similar to that of the non-encapsulated control strain (SS2Δ*cps*), whereas the well-encapsulated wild-type serotype 2 strain P1/7 showed very low hydrophobicity. As expected, the complemented SS2ΔsiaT mutant (SS2compΔsiaT) showed partially reduced hydrophobicity levels when compared to the SS2ΔsiaT mutant ($P = 0.0027$).

Despite the pMX1 vector was successfully used in several complementation studies, complementation in *S. suis* with pMX1 results in an intermediate state when compared to deficient mutant and wild-type strain (21, 22, 32). Absence of CPS expression by the SS2ΔsiaT mutant was also confirmed by TEM, as shown in Figure 4B, where a total loss of CPS expression was observed in the mutant strain compared to an apparent thick CPS surrounding the wild-type strain.

3.3. Substitution of the α-2,6-sialyltransferase by the GBS α-2,3-sialyltransferase in S. suis serotypes 2 and 14 also results in non-encapsulated phenotypes

In order to better dissect the importance and specificity of the sialyltransferase for CPS expression at the bacterial surface, we substituted the *S. suis* α-2,6-sialyltransferase by the GBS α-2,3-sialyltransferase using the same mutagenesis approach. The *S. suis* serotype 2 mutant SS2sia2,3 and the *S. suis* serotype 14 mutant SS14sia2,3 both showed very high hydrophobicity, which was similar to that of respective non-encapsulated mutant strains (Figure 5A). In addition, we investigated the presence of sialic acid and its linkage (if present) by an ELLA using α-2,6- or α-2,3-specific lectins. As shown in Figures 5B and 5C, *S. suis* serotype 2 and 14 mutants carrying the GBS α-2,3-sialyltransferase (SS2sia2,3 and SS14sia2,3) presented negative reactions with both SNA-I lectin (α-2,6) and MAL-I lectin (α-2,3), suggesting total absence of sialic acid at the bacterial surface. Consistent with hydrophobicity test results, TEM analysis confirmed the non-encapsulated phenotypes of mutants SS2sia2,3 and SS14sia2,3 (Figure 6).

3.4. Deletion of sialyltransferase or sialic acid synthase genes in GBS type V results in asialo encapsulated phenotype

In order to determine if the non-encapsulated phenotype resulting from deletion of the sialic acid synthesis gene or deletion/substitution of the sialyltransferase gene is specific to *S. suis,* we investigated for the first time the role of sialic acid in CPS expression by GBS type V. In contrast to *S. suis*, deletion of the sialyltransferase gene (*cps5K*) or the sialic acid synthesis gene (*neu5B*) of GBS type V had no inhibitory effect on CPS expression at the bacterial surface. Indeed, as shown in Figure 7A, the sialyltransferase mutant GBSVΔsiaT (Δ*cps5K*) and the sialic acid synthase mutant GBSVΔsynth (Δ*neu5B*) possessed moderate

hydrophobicity, which was indeed similar to that of the encapsulated wild-type strain and significantly lower to that obtained with the non-encapsulated type V mutant used as control (P = 0.0063 for GBSVΔsynth and P = 0.0019 for GBSVΔsiaT). These results suggest similar CPS expression between wild-type strain and both mutants (GBSVΔsiaT and GBSVΔsynth). The ELLA showed negative reactions with both SNA-I and MAL-I lectins (Figure 7B), suggesting total absence of sialic acid in the CPS produced by GBSVΔsiaT and GBSVΔsynth mutants. TEM analyses were used to confirm the presence of CPS in these two mutant strains. As depicted in Figure 8, mutants GBSVΔsiaT and GBSVΔsynth showed intermediate levels of encapsulation when compared to the serotype V wild-type strain and the non-encapsulated mutant GBSVΔ*cps*. Using the same CPS purification protocol for all strains, the CPS yield recovered from mutants GBSVΔsiaT and GBSVΔsynth was reduced when compared to wild-type GBS type V (Table 2), confirming intermediate levels of encapsulation in the two mutant strains. Analyses of purified CPSs by SEC-MALS also showed reduced weight-average molecular mass (M_w) for the two mutant strains derived CPSs when compared to the wild-type strain (Table 2).

Finally, we analyzed purified CPSs by NMR to confirm the absence of sialic acid in mutants GBSVΔsiaT and GBSVΔsynth. Integration of sialic acid reporter resonance signals (*i.e.*, H-3e at δ 2.76, H-3a at δ 1.77, and *N*-acetyl CH_3 at δ 2.03) in the ^{1}H spectrum of the GBSVΔsynth (Δ*neu5B*) mutant CPS (Figure 9B) represented *ca.* 0.2 equivalent compared to that of the wild-type GBS type V CPS (Figure 9A). These signals were totally absent from the spectrum of the GBSVΔsiaT (Δ*cps5K*) mutant CPS (Figure 9C), which in fact was essentially identical to that of the chemically desialylated GBS type V polysaccharide (Figure 9D) (29).

3.5. Substitution of the GBS α-2,3-sialyltransferase by the S. suis α-2,6-sialyltransferase in GBS results in successfully modified sialic acid linkage

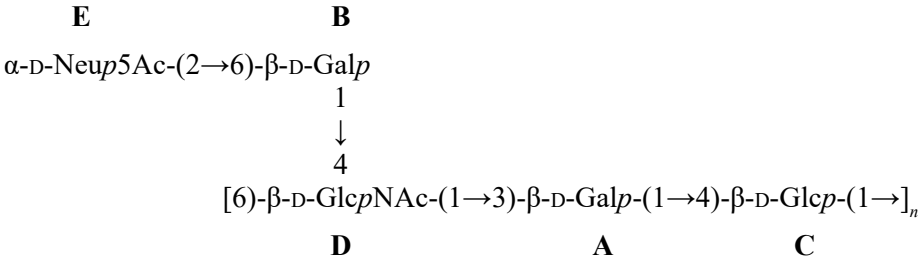
In order to study the specific role of sialic acid linkage in CPS expression by GBS, we substituted GBS type III and V sialyltransferase *cpsK* genes by the *S. suis* sialyltransferase gene *cpsN*. We first evaluated CPS expression by the hydrophobicity test. As shown in Figure 10A, mutants with modified sialyltransferase GBSIIIsia2,6 and GBSVsia2,6 presented high hydrophobicity when compared to the wild-type strain, but significantly lower than the non-

encapsulated mutant (P = 0.0473 for GBSIIIsia2,6 and P = 0.0046 for GBSVsia2,6), suggesting the presence of reduced amount of capsule at the bacterial surface. The ELLA (Figures 10B and 10C) was used to verify the sialic acid linkage obtained after sialyltransferase gene substitution. Mutants GBSIIIsia2,6 and GBSVsia2,6 showed positive reactions with SNA-I (α-2,6) lectin when compared to GBS wild-type strains (P = 0.0016 for GBSIIIsia2,6 and P = 0.0019 for GBSVsia2,6) (Figure 10B). In addition, negative reactions were observed for GBSIIIsia2,6 and GBSVsia2,6 with MAL-I (α-2,3) lectin, suggesting the expression of a CPS with modified sialic acid linkage (Figure 10C).

Further investigation of CPS expression at the bacterial surface was done by TEM, which showed a slim CPS surrounding the bacteria for mutants GBSIIIsia2,6 (Figure 11) and GBSVsia2,6 (Figure 8), whereas a thicker CPS was observed for respective wild-type strains. In contrast, non-encapsulated control strains GBSIIIΔ*cps* (Figure 11) and GBSVΔ*cps* (Figure 8) showed complete absence of CPS expression. Purified CPS yield recovered from mutants GBSIIIsia2,6 and GBSVsia2,6 was significantly reduced when compared to wild-type strains (Table 2), confirming intermediate levels of encapsulation. Analyses of purified CPSs by SEC-MALS also showed reduced weight-average molecular mass (M_w) for the two mutant strains derived CPSs (Table 2).

NMR analyses were used to confirm the nature of sialic acid linkage. Reporter resonance signals H-3e and H-3a were found at lower frequencies for the GBSIIIsia2,6 mutant compared to the wild-type GBS type III CPSs (Figure 12), consistent with sialic acid being 2,6- instead of 2,3-linked to the galactose residue (33). Since major differences were also visible in other spectral regions, complete structural analysis was performed using a series of 1D and 2D experiments. Residues were labelled A–D in order of increasing chemical shift of their anomeric protons. On the COSY spectrum, correlations from anomeric protons could be followed up to A4, B4, C3, and D2 and from E3 protons to E4 (Supplementary Figure S1). This was extended on the TOCSY spectrum (not shown) up to C5, D5, and E6, which confirmed the *galacto* configuration for residues A and B and the *gluco* configuration for residues C and D. Intra-residue correlations between axial protons in positions 1, 3, and 5 were observed on the ROESY spectrum (not shown) for residues A–D. Reporter resonances were

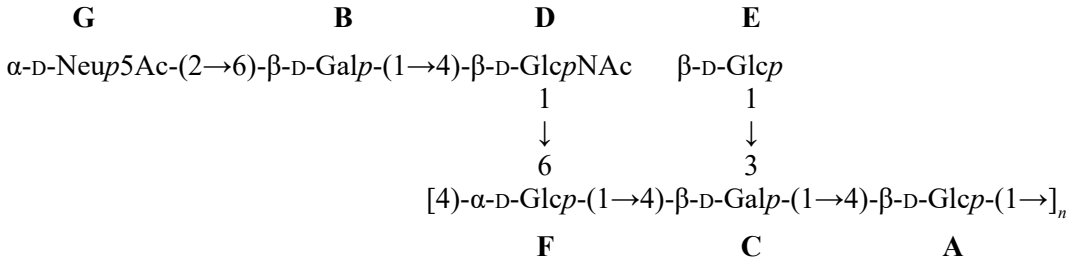
found on the ^{13}C spectrum: 3 carbonyl, 5 anomeric (Supplementary Figure S2 trace), 2 amino (Supplementary Figure S2 trace), 1 methylene of sialic acid, and 2 acetyl methyl carbons. The DEPT spectrum (Supplementary Figure S2 trace) confirmed a linkage at position 6 for two sugar residues. Carbons were assigned using the HSQC (Supplementary Figure S2) and HSQC–TOCSY (not shown) spectra, which also allowed identifying previously unassigned proton resonances. Full ^{1}H and ^{13}C assignments are listed in Supplementary Table S2. When compared to corresponding methyl glycosides, ^{13}C α glycosidation shifts of 2.32–9.62 ppm were observed for carbons A3, B6, C4, D4, and D6. On the ROESY spectrum (not shown), a correlation was observed between the *N*-acetyl CH_3 at δ 2.05 and D2. In addition, a few inter-residue correlations could readily be identified: C4–6/A1, D6/C1, A2–3/D1. Inter-residue correlations found on the HMBC spectrum (not shown), both from anomeric carbons (A1/C4, B1/D4, C1/D6', D1/A3, E2/B6, and E2/B6') and to anomeric protons (C4/A1, D4/B1, D6/C1, and A3/D1), confirmed the true linkage positions for all residues. Finally, the experiment also allowed assignments of acetyl carbons.



Scheme 1. Structure of the GBSIIIsia2,6 mutant CPS with residue labels.

Chemical shift differences between mutant and wild-type CPSs are reported in Supplementary Table S3. Significant differences (> 1 standard deviation) were found for protons B1, B3, B6, B6', D4, and E3a and for carbons B1, B2, B3, B5, B6, and D4. The transformation from 2,3- to 2,6-linked sialic acid was clearly evidenced by the large negative and positive ^{13}C chemical shift differences for B3 and B6, respectively. The large ^{1}H and ^{13}C chemical shift differences at D4 indicate that not only the presence of sialic acid (34), but also its linkage position exerts conformational control over the CPS backbone.

Similar differences in sialic acid ^{1}H reporter resonance signal positions were observed for the GBSVsia2,6 mutant compared to the wild-type GBS type V CPS (Figure 13), confirming the transformation to a 2,6-linkage for sialic acid in this serotype as well. Complete structural analysis was also performed for this CPS. Residues were labelled A–F in order of increasing chemical shift of their anomeric protons. On the COSY spectrum, correlations from anomeric protons could be followed up to A3, B3, C4, D2, E6, E6', and F2 and from G3 protons to G4 (Supplementary Figure S3). This was extended on the TOCSY spectrum (not shown) up to A4, B4, D5, F5, and G6, and the *galacto* configuration was confirmed for residues B and C and the *gluco* configuration for residue D. Intra-residue correlations between axial protons in positions 1, 3, and 5 were observed on the ROESY spectrum (not shown) for residues A–E. Reporter resonances were found on the ^{13}C spectrum: 3 carbonyl, 7 anomeric (Supplementary Figure S4 trace), 2 amino (Supplementary Figure S4 trace), 1 methylene of sialic acid, and 2 acetyl methyl carbons. The DEPT spectrum (Supplementary Figure S4 trace) confirmed a linkage at position 6 for two sugar residues. Carbons were assigned using the HSQC (Supplementary Figure S4) and HSQC–TOCSY spectra (not shown), which also allowed identifying previously unassigned proton resonances. Full ^{1}H and ^{13}C assignments are listed in Supplementary Table S4. When compared to corresponding methyl glycosides, ^{13}C α glycosidation shifts of 2.32–10.26 ppm were observed for carbons A4, B6, C3, C4, D4, F4, and F6. On the ROESY spectrum (not shown), a few inter-residue correlations could readily be identified: A1/F4, B1/D3–4, B1/D6', C1/A3–4, E1/C3, and F1/C4–5. Inter-residue correlations found on the HMBC spectrum (not shown), both from anomeric carbons (B1/D4, C1/A4, G2/B6, and G2/B6') and to anomeric protons (D4/B1, A4/C1, and C3/E1), confirmed the true linkage positions for several residues. Again, the experiment also allowed assignments of acetyl carbons.



Scheme 2. Structure of the GBSVsia2,6 mutant CPS with residue labels.

Only anomeric proton chemical shifts have been reported for the wild-type type V CPS (6), so most chemical shift differences, and as a consequence conformational control, cannot be evaluated in this case.

4. Discussion

This study dissected for the first time the effect of sialic acid synthesis, sialic acid linkage and sialyltransferase specificity on CPS expression by the only two so far described Gram-positive bacterial species able to sialylate their CPSs. We demonstrated that in spite of common CPS structural characteristics and similarities in the *cps* loci, sialic acid exerts differential control of CPS expression by *S. suis* and GBS.

The regulation of *S. suis* CPS synthesis is not clearly established. An interesting hypothesis is that *S. suis* may modulate the production of its CPS according to the environment. As a 'wrapping' surface component, decreased CPS thickness would promote interaction of different surface adhesins with their ligands and consequently increase adhesion to host epithelial cells during colonization. Once in the bloodstream, optimal CPS expression at the bacterial surface is required to resist immune-clearance. Yet, there is no evidence that *S. suis* actually modulates CPS thickness *in vivo*. Nevertheless, some regulators or proteins are able to influence *in vitro* the expression of CPS synthesis genes, such as the regulator protein of carbon metabolism Ccpa (35), the regulator CovR (36), the global regulator CodY (37) and the di-adenosine monophosphate phosphodiesterase (38). In addition, a study highlighting the role of a messenger RNA in the modulation of CPS synthesis has been recently published (39). In fact, *S. suis* CPS regulation may be subjected to several factors and/or environmental conditions.

Compared to GBS, CPS structures and *cps* loci have been less studied in *S. suis*. To date, there was no clear information about *cps* locus transcription for *S. suis*. In order to

investigate the presence of additional promoters within the *cps* locus of *S. suis*, RT-PCR analyses were applied and demonstrated that the *cps* locus is transcribed as a single polycistronic message, confirming that the *S. suis cps* locus is regulated from a single downstream promoter, as it was also described for GBS (40). Indeed, sialyltransferase and sialic acid synthesis genes are under the same promoter than the glycosyltransferase and CPS regulatory proteins. Giving the fact that GBS and *S. suis* sialyltransferases are regulated in the same manner, substitution between their sialyltransferase genes was thus conceivable.

Firstly, we investigated the role of the sialyltransferase in CPS expression by *S. suis*. It is already known that mutations in genes encoding regulatory enzymes (*cpsB* and *cpsD*), glycosyltransferases (*cpsE* and *cpsF*) and sialic acid synthesis pathway (*neuC*) result in a non-encapsulated phenotype for *S. suis* (21, 22, 41). Since several attempts in our laboratory to directly knockdown the sialyltransferase gene (*cps2N*) in the genome of *S. suis* serotype 2 failed due to mutation-induced lethality, confirming a previous report (19), we developed an indirect three-step mutagenesis approach based on double cross-over homologous recombination to delete *S. suis cps2N*. Indeed, mutations in side-chain formation (*cpsJ*), sialylation (*cpsN*), polymerase (*cpsL*), and flippase (*cpsO*) genes are lethal for *S. suis*, but also for other streptococci, as it is the case for *Streptococcus pneumoniae* (19, 42). In contrast, some of these mutations are known to be non-lethal for GBS type III, as it is the case for mutations in the sialyltransferase gene (*cpsK*) (43), suggesting differences in CPS synthesis between *S. suis* and GBS. The lethality of these mutations in *S. suis* and *S. pneumoniae* is hypothesized to be due to the sequestration of undecaprenyl-phosphate carrier CPS precursor in the incomplete CPS synthesis pathway that is also needed for other biological functions, such as synthesis of cell-wall peptidoglycan. However, a mutation in the *S. suis cpsN* gene can occur in presence of suppressor mutations in other CPS genes that inhibit CPS synthesis, as it was demonstrated with suppressor mutation in *cpsEF* (19). Giving the fact that deletion of the *neuC* gene also inhibits CPS synthesis (21), we used it as suppressor mutation in order to indirectly knockdown *cps2N*. Unfortunately, *S. suis* type 2 sialyltransferase gene deletion (SS2ΔsiaT) resulted in a non-encapsulated phenotype, confirming the critical role of sialic acid in the CPS expression by *S. suis*. Giving the fact that complementation of SS2ΔsiaT mutant restore CPS synthesis and no mutation was found in *cps2E* and *cps2F* genes (data not

shown), the resulting phenotype is most likely caused by the deletion of *cps2N*. Based on these results, one possible hypothesis is that the polymerase and/or the flippase enzymes of *S. suis* recognize the sialic acid moiety in the polysaccharide repeating unit in order to polymerize and/or export the CPS, respectively.

In contrast to *S. suis*, the presence of sialic acid in the polysaccharide repeating unit is not absolutely required for CPS expression by GBS, as sialyltransferase or sialic acid synthase gene deletion mutants of GBS type III (21, 43) and GBS type V (this work) are still able to express CPS at their surface. Yet, the obtained asialo-encapsulated mutants present reduced CPS amounts, suggesting that the sialic acid pathway is important, albeit not vital, for optimal CPS expression by GBS types III and V. The herein-observed low-encapsulation phenotype of GBSVΔsiaT and GBSVΔsynth mutants may result of reduced efficiency of the polymerase to polymerize the polysaccharides in absence of sialic acid. Indeed, SEC-MALS analyses of purified CPSs of mutants GBSVΔsiaT and GBSVΔsynth confirmed reduced CPS M_w compared to the wild-type CPS, suggesting that sialic acid is necessary for optimal CPS polymerization. This is in contrast to results reported by (43), where the GBS type III asialo mutant (Δ*cpsK*) shows longer polysaccharide chain length than the wild-type sialyled CPS. Indeed, for GBS type III, reduced CPS amounts in the asialo mutant (Δ*cpsK*) do not seem to be related to shorter polysaccharide chains, but is likely due to reduced transfer of CPS precursors across the cytoplasmic membrane (43). Our results with GBSVΔsiaT and GBSVΔsynth mutants indicate that differences exist between these two serotypes in how they behave in terms of CPS polymerization and export in the absence of sialic acid. In spite of these inter-serotype differences, when compared to *S. suis*, overall GBS polymerase and/or flippase seem to be more versatile and less specific for substrate than the *S. suis* respective enzymes.

To evaluate how the specificity of the sialyltransferase and the resulting sialic acid linkage affect CPS expression, we performed for the first time inter-species sialyltransferase exchange between these two Gram-positive bacterial species. Since aforementioned differences between GBS types III and V, the two serotypes were analyzed and compared to two different serotypes (2 and 14) of *S. suis*. Substitution of the *S. suis* α-2,6-sialyltransferase

by the GBS α-2,3-sialyltransferase in *S. suis* serotypes 2 and 14 also results in a non-encapsulated phenotype. This phenotype might be related to a high specificity of *S. suis* polymerase and/or flippase for α-2,6 terminal sialic acid in both *S. suis* serotypes. It can be hypothesized that *S. suis* polysaccharide chains need to be α-2,6-sialylated in order to be recognized by *S. suis* polymerase and/or flippase enzymes. Another hypothesis is that the GBS α-2,3 sialyltransferases are specific to GBS polysaccharide structures and thus unable to recognize *S. suis* polysaccharide chain/structure in order to transfer sialic acid to the terminal galactose, consequently inhibiting polymerization and/or exportation of the CPS. In fact, GBS type III and *S. suis* sialyltransferases share only 33% of protein identity. However, the sialyltransferase gene is 100% identical between *S. suis* serotypes 2 (strain P1/7) and 14 (strain DAN13730) and highly conserved among other sialylated *S. suis* serotypes, such as serotypes 1 and 1/2, suggesting that the *S. suis* sialyltransferase may recognize a common epitope/component (5). In contrast, GBS sialyltransferases of types III and V express significant differences in the 5' region of the *cpsK* gene and may recognize different epitopes/components (43). In this study, we used the GBS type III sialyltransferase in *S. suis*, thus we cannot rule out the possibility that the GBS type V sialyltransferase may be able to sialylate *S. suis* polysaccharides due to the differences between type III and V sialyltransferases (43).

In contrat to *S. suis*, GBS type III and V mutants carrying the α-2,6-sialyltransferase of *S. suis* are still able to express CPS at the bacterial surface, albeit at a reduced amount. These results support the hypothesis that GBS polymerase and/or flippase seem to be more versatile than those of *S. suis*. As such, modification of sialic acid linkage (α-2,3 to α-2,6) affects, but not completely inhibits, recognition of polysaccharide subunits/chain by polymerase and/or flippase and consequently leads to a CPS of reduced thickness and a diminished polysaccharide chain length (represented by M_w) in both mutants. Importantly, we demonstrated by 1D and 2D NMR spectroscopy that the overall CPS structure of GBS types III and V was preserved in GBSIIIsia2,6 and GBSVsia2,6 mutants, respectively, and that only the linkage between sialic acid and side-chain galactose was effectively changed from α-2,3 to α-2,6 as expected. The *S. suis* serotype 2 α-2,6-sialyltransferase probably recognizes common pattern(s) shared by both *S. suis* and GBS in CPS components/structure. This is the first time

that exogenous sialyltransferase replacement is used in order to express CPSs with modified sialic acid linkage in Gram-positive bacteria.

The nature of carbohydrate epitopes, such as those present in bacterial CPSs, is diverse and can be linear or conformational. Serological analyses have suggested that the side chain, and more particularly the terminal sialic acid, constitutes one important epitope for *S. suis* serotype 2 (44). Nevertheless, the α-2,6 substitution in GBS types III and V CPSs failed to confer immunological cross-reaction with anti-*S. suis* type 2 or type 14 CPS antibodies (unpublished observations), highlighting the complexity of these carbohydrate epitopes.

In conclusion, a critical role of sialic acid (and its linkage) in *S. suis* CPS expression at the bacterial surface was demonstrated. Unfortunately, the non-encapsulated phenotype makes impossible so far to study the precise role of sialic acid in *S. suis* pathogenesis. In contrast, GBS is able to express asialo CPS or α-2,6-sialylated CPS, although the amount of polysaccharide at the bacterial surface is reduced. Albeit this limited expression of CPS at the bacterial surface might compromise accurate studies on the role of sialic acid linkage in host-pathogen interactions, the modified GBS type III and V CPSs represent new tools to study CPS immunogenicity and biochemistry.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a grant to MS (#342150) with partial contribution of NSERC grant to MG (#04146). DR is the recipient of a Fonds de recherche du Québec—Nature et technologies (FRQ-NT) Doctoral Award; GGD is the recipient of a FRQ-NT Doctoral Award and an Alexander Graham Bell Graduate Scholarship - Doctoral Program from NSERC. DR and GGD are also recipients of scholarships from the Swine and Poultry Infectious Disease Research Center.

References

1. Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, et al. Structural and genetic diversity of group B streptococcus capsular polysaccharides. Infection and immunity. 2005;73(5):3096-103.
2. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. Streptococcus suis, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerging microbes & infections. 2014;3(6):e45.
3. Johri AK, Paoletti LC, Glaser P, Dua M, Sharma PK, Grandi G, et al. Group B Streptococcus: global incidence and vaccine development. Nature reviews Microbiology. 2006;4(12):932-42.
4. Madzivhandila M, Adrian PV, Cutland CL, Kuwanda L, Schrag SJ, Madhi SA. Serotype distribution and invasive potential of group B streptococcus isolates causing disease in infants and colonizing maternal-newborn dyads. PloS one. 2011;6(3):e17861.
5. Okura M, Takamatsu D, Maruyama F, Nozawa T, Nakagawa I, Osaki M, et al. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of Streptococcus suis: potential mechanisms for generation of capsular variation. Applied and environmental microbiology. 2013;79(8):2796-806.
6. Wessels MR, DiFabio JL, Benedi VJ, Kasper DL, Michon F, Brisson JR, et al. Structural determination and immunochemical characterization of the type V group B Streptococcus capsular polysaccharide. The Journal of biological chemistry. 1991;266(11):6714-9.
7. Wessels MR, Kasper DL. Correct structure of repeating unit of group B Streptococcus type III capsular polysaccharide. The Journal of infectious diseases. 1990;162(6):1412.
8. Wessels MR, Pozsgay V, Kasper DL, Jennings HJ. Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III group B Streptococcus. A revised structure for the type III group B streptococcal polysaccharide antigen. The Journal of biological chemistry. 1987;262(17):8262-7.
9. Van Calsteren MR, Gagnon F, Calzas C, Goyette-Desjardins G, Okura M, Takamatsu D, et al. Structure determination of Streptococcus suis serotype 14 capsular polysaccharide. Biochemistry and cell biology = Biochimie et biologie cellulaire. 2013;91(2):49-58.
10. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of Streptococcus suis serotype 2 capsular polysaccharide. Biochemistry and cell biology = Biochimie et biologie cellulaire. 2010;88(3):513-25.
11. Chabot-Roy G, Willson P, Segura M, Lacouture S, Gottschalk M. Phagocytosis and killing of Streptococcus suis by porcine neutrophils. Microbial pathogenesis. 2006;41(1):21-32.
12. Charland N, Harel J, Kobisch M, Lacasse S, Gottschalk M. Streptococcus suis serotype 2 mutants deficient in capsular expression. Microbiology. 1998;144 (Pt 2):325-32.
13. Lecours MP, Gottschalk M, Houde M, Lemire P, Fittipaldi N, Segura M. Critical role for Streptococcus suis cell wall modifications and suilysin in resistance to complement-dependent killing by dendritic cells. The Journal of infectious diseases. 2011;204(6):919-29.

14. Lecours MP, Segura M, Lachance C, Mussa T, Surprenant C, Montoya M, et al. Characterization of porcine dendritic cell response to Streptococcus suis. Veterinary research. 2011;42:72.
15. Lemire P, Roy D, Fittipaldi N, Okura M, Takamatsu D, Bergman E, et al. Implication of TLR- but not of NOD2-signaling pathways in dendritic cell activation by group B Streptococcus serotypes III and V. PloS one. 2014;9(12):e113940.
16. Segura M. Fisher scientific award lecture - the capsular polysaccharides of Group B Streptococcus and Streptococcus suis differently modulate bacterial interactions with dendritic cells. Canadian journal of microbiology. 2012;58(3):249-60.
17. Segura MA, Cleroux P, Gottschalk M. Streptococcus suis and group B Streptococcus differ in their interactions with murine macrophages. FEMS immunology and medical microbiology. 1998;21(3):189-95.
18. Bax M, Kuijf ML, Heikema AP, van Rijs W, Bruijns SC, Garcia-Vallejo JJ, et al. Campylobacter jejuni lipooligosaccharides modulate dendritic cell-mediated T cell polarization in a sialic acid linkage-dependent manner. Infection and immunity. 2011;79(7):2681-9.
19. Lakkitjaroen N, Takamatsu D, Okura M, Sato M, Osaki M, Sekizaki T. Capsule loss or death: the position of mutations among capsule genes sways the destiny of Streptococcus suis. FEMS microbiology letters. 2014;354(1):46-54.
20. Warrens AN, Jones MD, Lechler RI. Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest. Gene. 1997;186(1):29-35.
21. Lecours MP, Fittipaldi N, Takamatsu D, Okura M, Segura M, Goyette-Desjardins G, et al. Sialylation of Streptococcus suis serotype 2 is essential for capsule expression but is not responsible for the main capsular epitope. Microbes and infection. 2012;14(11):941-50.
22. Roy D, Auger JP, Segura M, Fittipaldi N, Takamatsu D, Okura M, et al. Role of the capsular polysaccharide as a virulence factor for Streptococcus suis serotype 14. Canadian journal of veterinary research = Revue canadienne de recherche veterinaire. 2015;79(2):141-6.
23. Roy D, Grenier D, Segura M, Mathieu-Denoncourt A, Gottschalk M. Recruitment of Factor H to the Streptococcus suis Cell Surface is Multifactorial. Pathogens. 2016;5(3).
24. Takamatsu D, Osaki M, Sekizaki T. Construction and characterization of Streptococcus suis-Escherichia coli shuttle cloning vectors. Plasmid. 2001;45(2):101-13.
25. Bonifait L, Gottschalk M, Grenier D. Cell surface characteristics of nontypeable isolates of Streptococcus suis. FEMS microbiology letters. 2010;311(2):160-6.
26. Geisler C, Jarvis DL. Effective glycoanalysis with Maackia amurensis lectins requires a clear understanding of their binding specificities. Glycobiology. 2011;21(8):988-93.
27. Shibuya N, Goldstein IJ, Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ. The elderberry (Sambucus nigra L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. The Journal of biological chemistry. 1987;262(4):1596-601.
28. Gornik O, Lauc G. Enzyme linked lectin assay (ELLA) for direct analysis of transferrin sialylation in serum samples. Clinical biochemistry. 2007;40(9-10):718-23.
29. Calzas C, Goyette-Desjardins G, Lemire P, Gagnon F, Lachance C, Van Calsteren MR, et al. Group B Streptococcus and Streptococcus suis capsular polysaccharides induce

chemokine production by dendritic cells via Toll-like receptor 2- and MyD88-dependent and -independent pathways. Infection and immunity. 2013;81(9):3106-18.
30. Wishart DS, Bigam CG, Yao J, Abildgaard F, Dyson HJ, Oldfield E, et al. 1H, 13C and 15N chemical shift referencing in biomolecular NMR. Journal of biomolecular NMR. 1995;6(2):135-40.
31. Zhu GB, A. Improved linear prediction of damped NMR signals using modified "forward-backward" linear prediction. Journal of Magnetic Resonance. 1992;100(1):202-7.
32. Ferrando ML, Willemse N, Zaccaria E, Pannekoek Y, van der Ende A, Schultsz C. Streptococcal Adhesin P (SadP) contributes to Streptococcus suis adhesion to the human intestinal epithelium. PloS one. 2017;12(4):e0175639.
33. Machytka D, Klein RA, Egge H. Reporter resonances in the NMR spectra of oligosaccharides containing sialic acid linked to galactopyranose rings. Carbohydrate research. 1994;254:289-94.
34. Brisson JR, Uhrinova S, Woods RJ, van der Zwan M, Jarrell HC, Paoletti LC, et al. NMR and molecular dynamics studies of the conformational epitope of the type III group B Streptococcus capsular polysaccharide and derivatives. Biochemistry. 1997;36(11):3278-92.
35. Willenborg J, Fulde M, de Greeff A, Rohde M, Smith HE, Valentin-Weigand P, et al. Role of glucose and CcpA in capsule expression and virulence of Streptococcus suis. Microbiology. 2011;157(Pt 6):1823-33.
36. Pan X, Ge J, Li M, Wu B, Wang C, Wang J, et al. The orphan response regulator CovR: a globally negative modulator of virulence in Streptococcus suis serotype 2. Journal of bacteriology. 2009;191(8):2601-12.
37. Feng L, Zhu J, Chang H, Gao X, Gao C, Wei X, et al. The CodY regulator is essential for virulence in Streptococcus suis serotype 2. Scientific reports. 2016;6:21241.
38. Du B, Ji W, An H, Shi Y, Huang Q, Cheng Y, et al. Functional analysis of c-di-AMP phosphodiesterase, GdpP, in Streptococcus suis serotype 2. Microbiological research. 2014;169(9-10):749-58.
39. Xiao G, Tang H, Zhang S, Ren H, Dai J, Lai L, et al. Streptococcus suis small RNA rss04 contributes to the induction of meningitis by regulating capsule synthesis and by inducing biofilm formation in a mouse infection model. Veterinary microbiology. 2017;199:111-9.
40. Chaffin DO, Beres SB, Yim HH, Rubens CE. The serotype of type Ia and III group B streptococci is determined by the polymerase gene within the polycistronic capsule operon. Journal of bacteriology. 2000;182(16):4466-77.
41. Smith HE, Damman M, van der Velde J, Wagenaar F, Wisselink HJ, Stockhofe-Zurwieden N, et al. Identification and characterization of the cps locus of Streptococcus suis serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infection and immunity. 1999;67(4):1750-6.
42. Xayarath B, Yother J. Mutations blocking side chain assembly, polymerization, or transport of a Wzy-dependent Streptococcus pneumoniae capsule are lethal in the absence of suppressor mutations and can affect polymer transfer to the cell wall. Journal of bacteriology. 2007;189(9):3369-81.

43. Chaffin DO, Mentele LM, Rubens CE. Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. Journal of bacteriology. 2005;187(13):4615-26.
44. Van Calsteren MR, Goyette-Desjardins G, Gagnon F, Okura M, Takamatsu D, Roy R, et al. Explaining the Serological Characteristics of Streptococcus suis Serotypes 1 and 1/2 from Their Capsular Polysaccharide Structure and Biosynthesis. The Journal of biological chemistry. 2016;291(16):8387-98.
45. Casadaban MJ, Cohen SN. Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. Journal of molecular biology. 1980;138(2):179-207.
46. Slater JD, Allen AG, May JP, Bolitho S, Lindsay H, Maskell DJ. Mutagenesis of Streptococcus equi and Streptococcus suis by transposon Tn917. Veterinary microbiology. 2003;93(3):197-206.
47. Gottschalk M, Higgins R, Jacques M, Mittal KR, Henrichsen J. Description of 14 new capsular types of Streptococcus suis. Journal of clinical microbiology. 1989;27(12):2633-6.
48. Takamatsu D, Osaki M, Sekizaki T. Thermosensitive suicide vectors for gene replacement in Streptococcus suis. Plasmid. 2001;46(2):140-8.

Tables

Table 1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	General Characteristics	Source/ Reference
Escherichia coli		
TOP 10	F-mrcA Δ(mrr-hsdRMS-mcrBC)ϕ80 lacZΔM5 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
MC1061	*araD139 Δ*(*ara-leu*)*7697 Δlac*X74 *galU galK hsdR2*(rK-mK+) *mcrB1 rpsL*	(45)
Streptococcus suis (SS) serotype 2		
SS2 WT	P1/7 wild type (WT), highly encapsulated serotype 2 strain isolated from a clinical case of swine infection in the United Kingdom	(46)
SS2Δ*cps*	Non-encapsulated isogenic mutant strain derived from strain P1/7. In-frame deletion of the *cps2F* gene (rhamnosyltransferase; locus tag: SSU0520)	(13)
SS2ΔsiaT	Isogenic mutant strain derived from strain P1/7. Indirect in-frame deletion of the *cps2N* gene (sialyltransferase; locus tag: SSU0533)	This work
SS2compΔsiaT	Mutant SS2ΔsiaT complemented with pMX1*cps2N*	This work
SS2sia2,3	Isogenic mutant strain derived from strain P1/7. Indirect substitution of the *cps2N* gene by the *cps3K* gene of GBS type III (sialyltransferase; locus tag: GBSCOH1_1070)	This work
SS2Δsynth	Non-encapsulated isogenic mutant strain derived from strain P1/7. In-frame deletion of the *neu2C* gene (UDP-*N*-acetylglucosamine 2-epimerase; locus tag: SSU0536)	(13)
SS2Δsynth/ΔsiaT	Isogenic mutant strain derived from strain P1/7. In-frame deletions of the *neu2C* and *cps2N* genes	This work
SS2Δsynth/sia2,3	Isogenic mutant strain derived from strain P1/7. In-frame deletion of the *neu2C* gene and substitution of the *cps2N* gene by the *cps3K* gene of GBS type III	This work

Streptococcus suis (SS) serotype 14		
SS14 WT	DAN13730 wild type (WT), highly encapsulated serotype 14 strain isolated from a human case in the Netherlands	(47)
SS14Δ*cps*	Non-encapsulated isogenic mutant strain derived from strain DAN13730. In-frame deletion of the *cps14B* gene (chain length determinant protein Wzd; locus tag: equivalent of SSU0516 in P1/7)	(22)
SS14sia2,3	Isogenic mutant strain derived from strain DAN13730. Indirect substitution of the sialyltransferase *cps14N* gene (locus tag: equivalent of SSU0533 in P1/7) by the *cps3K* gene of GBS type III	This work
SS14Δsynth	Non-encapsulated isogenic mutant strain derived from strain DAN13730. In-frame deletion of the *neu14C* gene (UDP-*N*-acetylglucosamine 2-epimerase; locus tag: equivalent of SSU0536 in P1/7)	(22)
SS14Δsynth/sia2,3	Isogenic mutant strain derived from strain DAN13730. In-frame deletion of the *neu14C* gene and substitution of the *cps14N* gene by the *cps3K* gene of GBS type III	This work
Group B *Streptococcus* (GBS) serotype III		
GBSIII WT	COH1 wild type (WT), well encapsulated type III strain isolated from an infant with sepsis and meningitis	(43)
GBSIIIsia2,6	Isogenic mutant strain derived from strain COH1. Direct substitution of sialyltransferase *cps3K* gene by *cps2N* gene of *S. suis* type 2	This work
GBSIIIΔ*cps*	Non-encapsulated isogenic mutant strain derived from strain COH1. In-frame deletion of the *cpsE* gene (glucosyltransferase; locus tag: GBSCOH1_1075)	(21)
GBSIIIΔsynth	Intermediate-encapsulated isogenic mutant strain derived from strain COH1. In-frame deletion of the *neu3B* gene (sialic acid synthase; locus tag: GBSCOH1_1066)	(21)

Group B *Streptococcus* (GBS) serotype V		
GBSV WT	CJB111 wild type (WT), well encapsulated type V strain isolated from neonate with septicemia	ATCC
GBSVsia2,6	Isogenic mutant strain derived from strain CJB111. Direct substitution of *cps5K* gene (sialyltransferase; locus tag: SAG1163) by *cps2N* gene of *S. suis* type 2	This work
GBSVΔsynth	Isogenic mutant strain derived from strain CJB111. In-frame deletion of the *neu5B* gene (sialic acid synthase; locus tag: SAG1161)	This work
GBSVΔsiaT	Isogenic mutant strain derived from strain CJB111. In-frame deletion of the *cps5K* gene	This work
GBSVΔ*cps*	Non-encapsulated isogenic mutant strain derived from strain CJB111. In-frame deletion of the *cpsE* gene (glucosyltransferase; locus tag: SAG1171)	(15)
Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Invitrogen
pSET4s	Thermosensitive vector for allelic replacement. Replication functions of pG+host3, MCS *oriR* pUC19 *lacZ* Sp^R	(48)
pMX1	Replication functions of pSSU1, MCS pUC19 lacZ Sp^R, malQ promoter of *S. suis,* derivative of pSET2	(48)
p4Δ*neuC*	pSET4s carrying the construct for *neuC* allelic replacement (*S. suis* serotypes 2 and 14)	This work
p4Δ*cps2N*	pSET4s carrying the construct for *cps2N* indirect allelic replacement	This work
p4*neuC*	pSET4s carrying intact *neuC* gene for *neuC* reintroduction (*S. suis* serotype 2 and 14)	This work
p4sia2,3_2	pSET4s carrying construct for allelic replacement of *S. suis cps2N* gene by *cps3K* from GBS type III	This work
p4sia2,3_14	pSET4s carrying construct for allelic replacement of *S. suis cps14N* gene by *cps3K* from GBS type III	This work
p4sia2,6_III	pSET-4s carrying construct for allelic replacement of GBS type III *cps3K* gene by *cps2N* from *S. suis* type 2	This work

p4sia2,6_V	pSET4s carrying construct for allelic replacement of GBS type V *cps5K* gene by *cps2N* from *S. suis* type 2	This work
p4Δ*neu5B*	pSET4s carrying construct for allelic deletion of GBS type V *neu5B* gene	This work
p4Δ*cps5K*	pSET4s carrying construct for allelic deletion of GBS type V *cps5K* gene	This work
pMX1*cps2N*	pMX1 complementation vector carrying intact *cps2N* gene	This work

Table 2. Group B *Streptococcus* (GBS) capsular polysaccharide (CPS) yields and weight-average molecular mass.

GBS strain	CPS type	Average yield (mg)	M_w (kg/mol)[a]
GBS III wild type	III	50.4[b]	108.0[b]
GBSIIIsia2,6	III	12.0	44.7
GBS V wild type	V	50.5[b]	128.0[b]
GBSVsia2,6	V	15.0	69.4
GBSΔsiaT	V	7.0	58.1
GBSΔsynth	V	16.0	62.4

[a] M_w, *weight-average molecular mass, determined by size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS).*

[b] *Published in Calzas et al. (2013). Infect. Immun. 81, 3106-3118.*

Figures

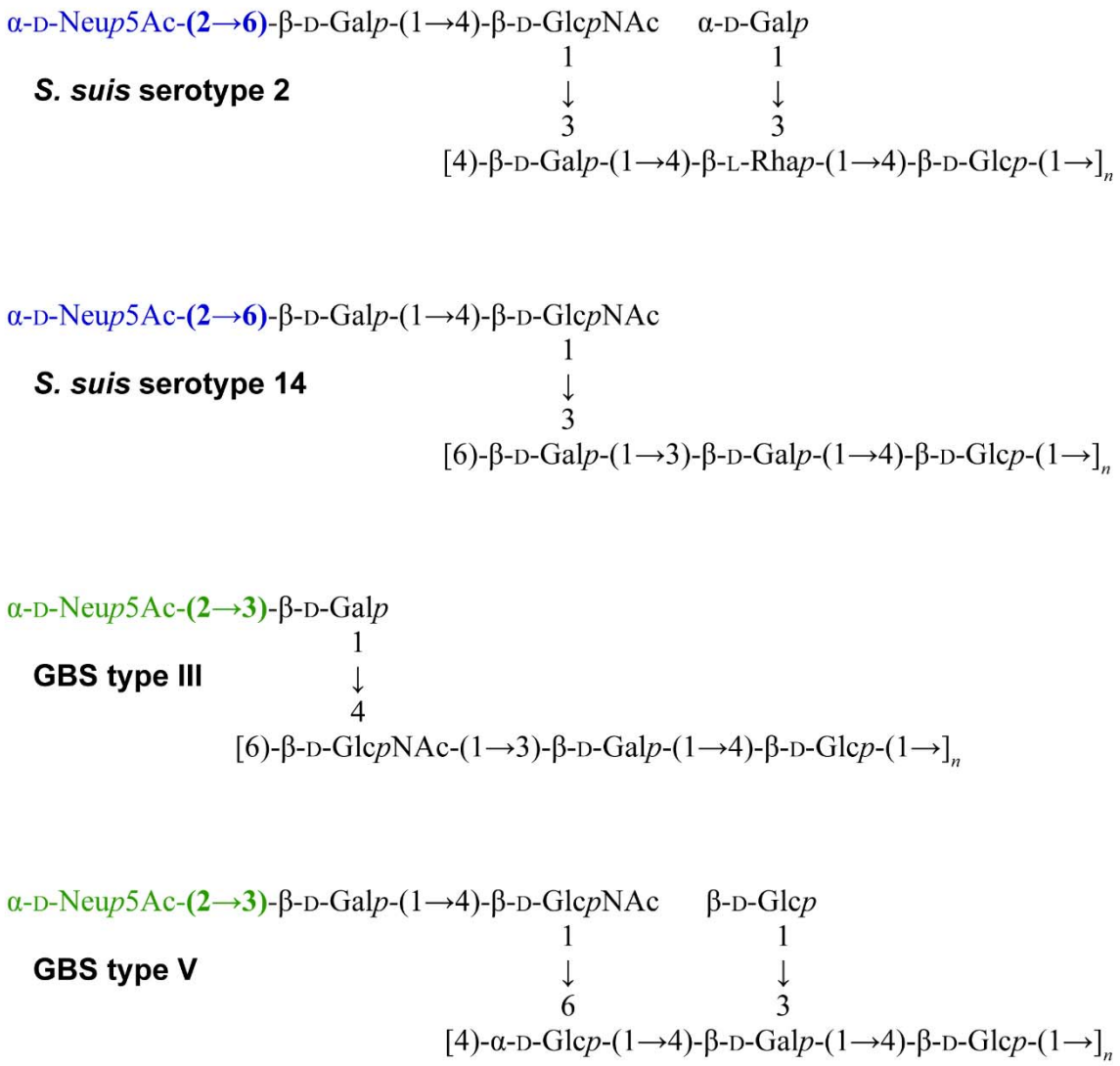


Figure 1. Reported structures for the capsular polysaccharide repeating units of *S. suis* serotypes 2 (10) and 14 (9) and of Group B *Streptococcus* (GBS) types III (6, 8) and V (6).

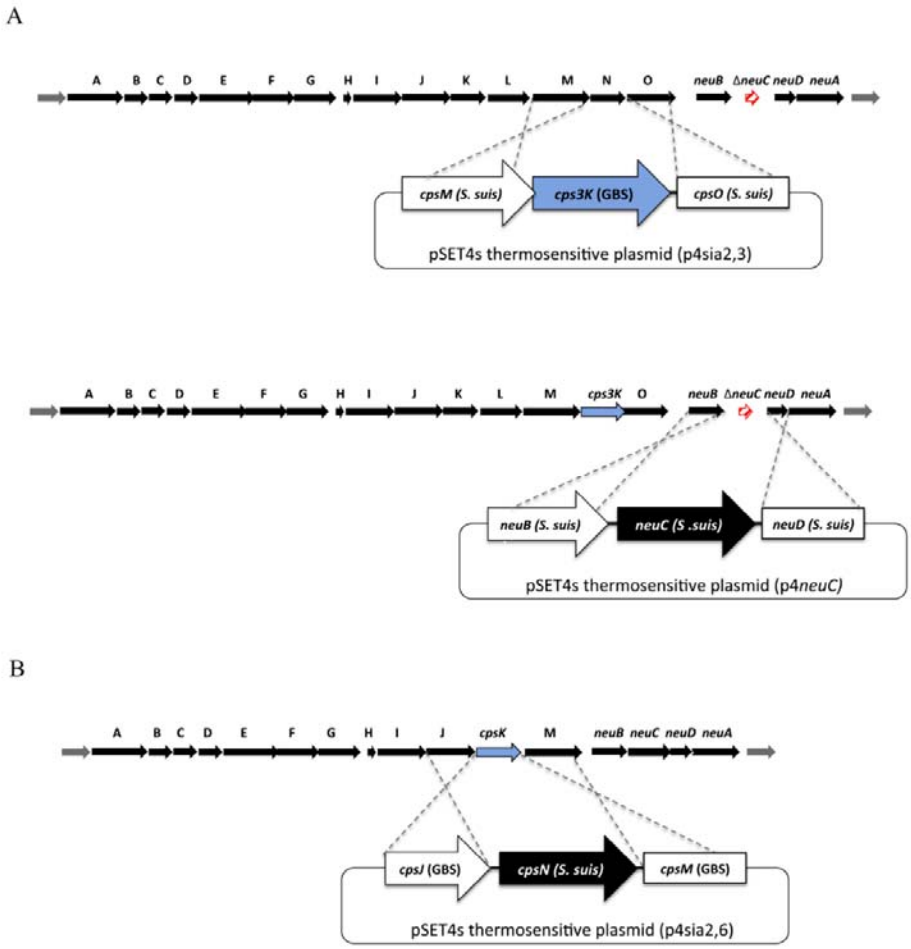


Figure 2. Schematic representation of mutagenesis procedures to obtain sialyltransferase substitution mutants in (A) *S. suis* and (B) Group B *Streptococcus* (GBS).

(A; top part) Non-lethal mutation in *neuC* (Δsynth) was used first to abolish capsular polysaccharide (CPS) production. Gene substitution plasmids p4sia2,3_2 and p4sia2,3_14 were introduced into *S. suis* SS2Δsynth (serotype 2) and SS14Δsynth (serotype 14) mutants, respectively. (A; bottom part) In order to reintroduce a functional *neuC* gene into SS2Δ*neu2C*/*cps3K* and SS14Δ*neu14C*/*cps3K* mutants, the p4*neuC* plasmid was introduced into SS2Δ*neu2C*/*cps3K* and SS14Δ*neu14C*/*cps3K* mutants to obtain indirect substitution mutants SS2sia2,3 and SS14sia2,3. (B) In order to substitute GBS type III and V sialyltransferases, direct gene replacement by double-crossover homologous recombination system was used by introducing mutation plasmids p4sia2,6_III and p4sia2,6_V.

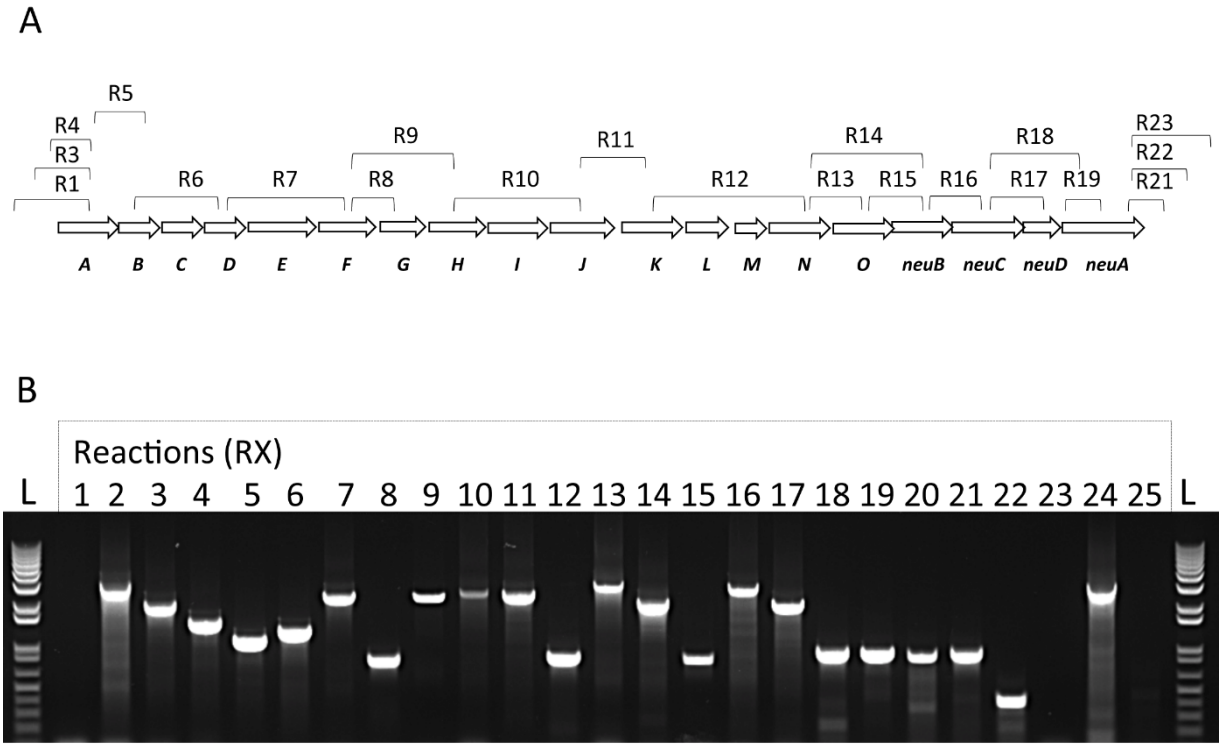
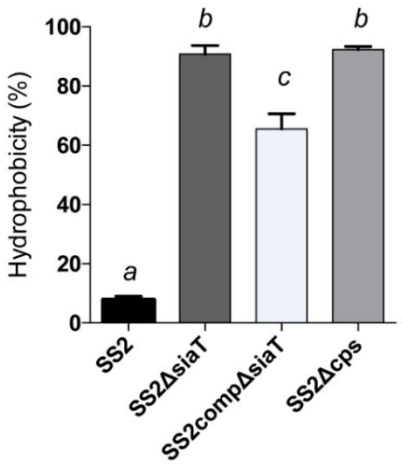


Figure 3. Transcriptional analysis of the *S. suis cps* operon.

(A) Schematic representation of *S. suis* capsular polysaccharide synthesis coding region with corresponding RT-PCR reactions and (B) agarose gel electrophoresis of RT-PCR reaction products visualized under UV light. Total bacterial RNA was isolated and converted to cDNA. RNA samples without reverse-transcription step were used as template to verify absence of genomic DNA in lane R25. Genomic DNA with the same primers was used as positive control (lane R24). All RT-PCR products migrated accordingly to their expected sizes. DNA size standards ("L") are depicted at the left- and right-end-sides.

A



B

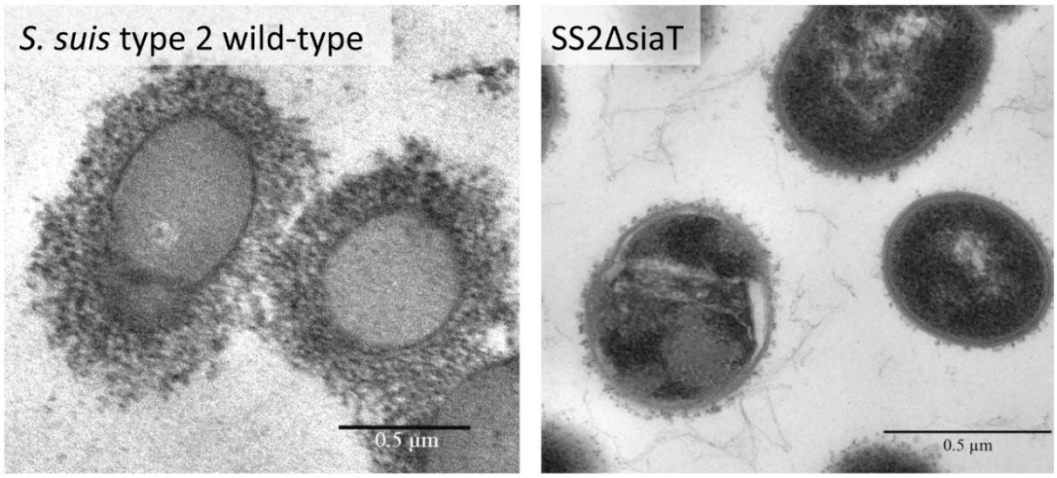


Figure 4. Capsular polysaccharide (CPS) expression levels of *S. suis* serotype 2 and derived isogenic mutants.

(A) Hydrophobicity (%) of the wild-type *S. suis* serotype 2 strain (SS2), the sialyltransferase SS2ΔsiaT (Δ*cps2N*) mutant, the complemented SS2compΔsiaT mutant, and of the non-encapsulated strain (SS2Δ*cps*) used as control. Data are expressed as mean ± SEM of at least three independent experiments. Student *t*-test analyses reported significant differences between *'a'* and *'b'*, between '*a'* and '*c'*, and between '*b'* and '*c'* ($P < 0.05$). (B) Transmission electron micrographs showing CPS expression by *S. suis* serotype 2 wild-type strain and its sialyltransferase SS2ΔsiaT (Δ*cps2N*) mutant. Bars = 0.5 µm.

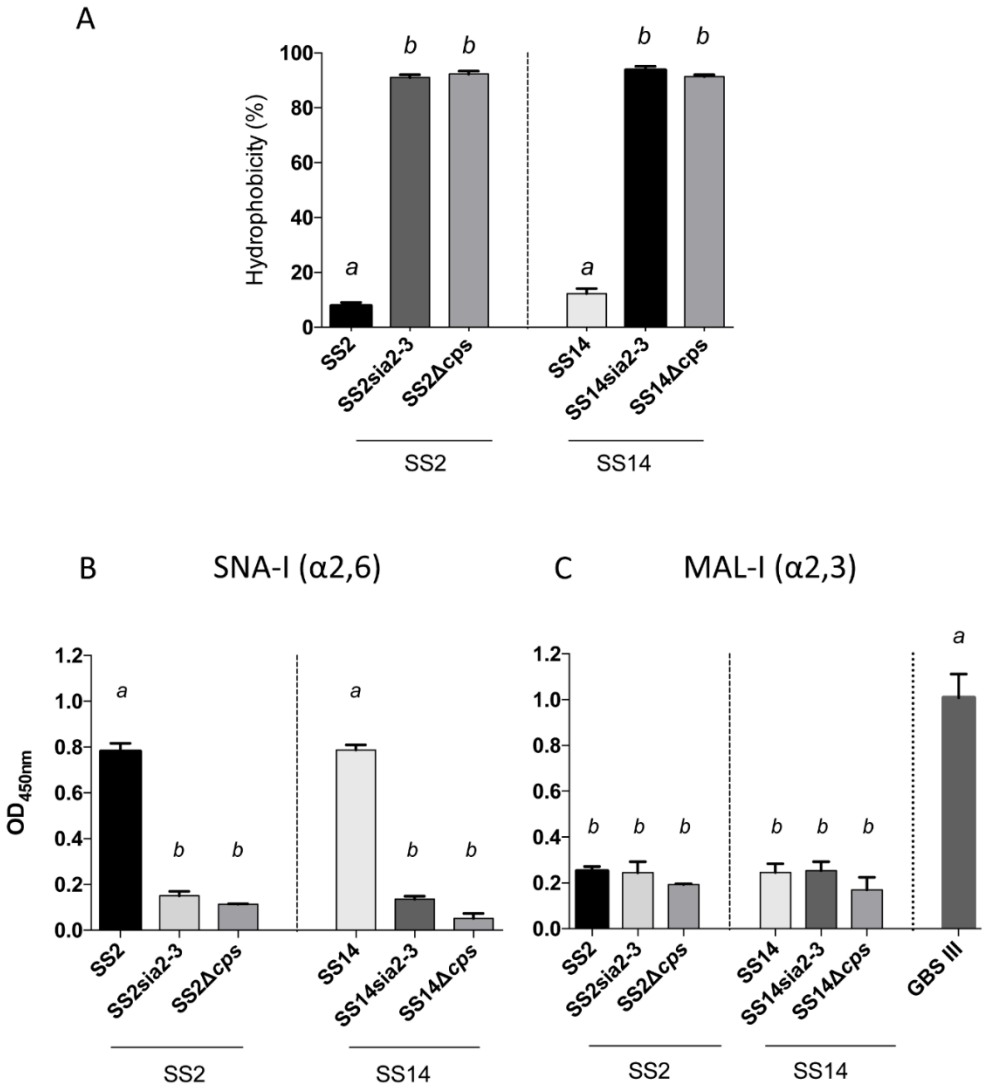


Figure 5. Capsular polysaccharide (CPS) expression levels and sialic acid linkage in *S. suis* serotype 2 and 14 mutants carrying exogenous α-2,3-sialyltransferase.

(A) Hydrophobicity (%) of the wild-type *S. suis* serotypes 2 (SS2) and 14 (SS14) strains, the SS2sia2,3 (Δ*cps2N*/*cpsK*) and SS14sia2,3 (Δ*cps14N*/*cpsK*) mutants carrying the GBS α-2,3-sialyltransferase (*cpsK*). The non-encapsulated mutants SS2Δ*cps* and SS14Δ*cps* were used as control strains. (B-C) Whole-bacterial cell enzyme-linked lectin assay (ELLA) was performed to detect α-2,3 or α-2,6 capsular sialic acid linkage in SS2sia2,3 and SS14sia2,3 mutant strains. Whole bacteria were incubated with *Sambucus nigra* agglutinin (SNA-I) specific for Neu5Ac α-2,6 linkages, or *Maackia amurensis* leukoagglutinin (MAL-I) specific for Neu5Ac α-2,3 linkages. The non-encapsulated mutants SS2Δ*cps* and SS14Δ*cps* were used as negative

controls. SS2 was used as positive control for SNA-I and wild-type GBS type III as positive control for MAL-I. Data in (A), (B), and (C) are expressed as mean ± SEM of at least three independent experiments. Student *t*-test analyses reported significant differences between '*a*' and '*b*' ($P < 0.05$).

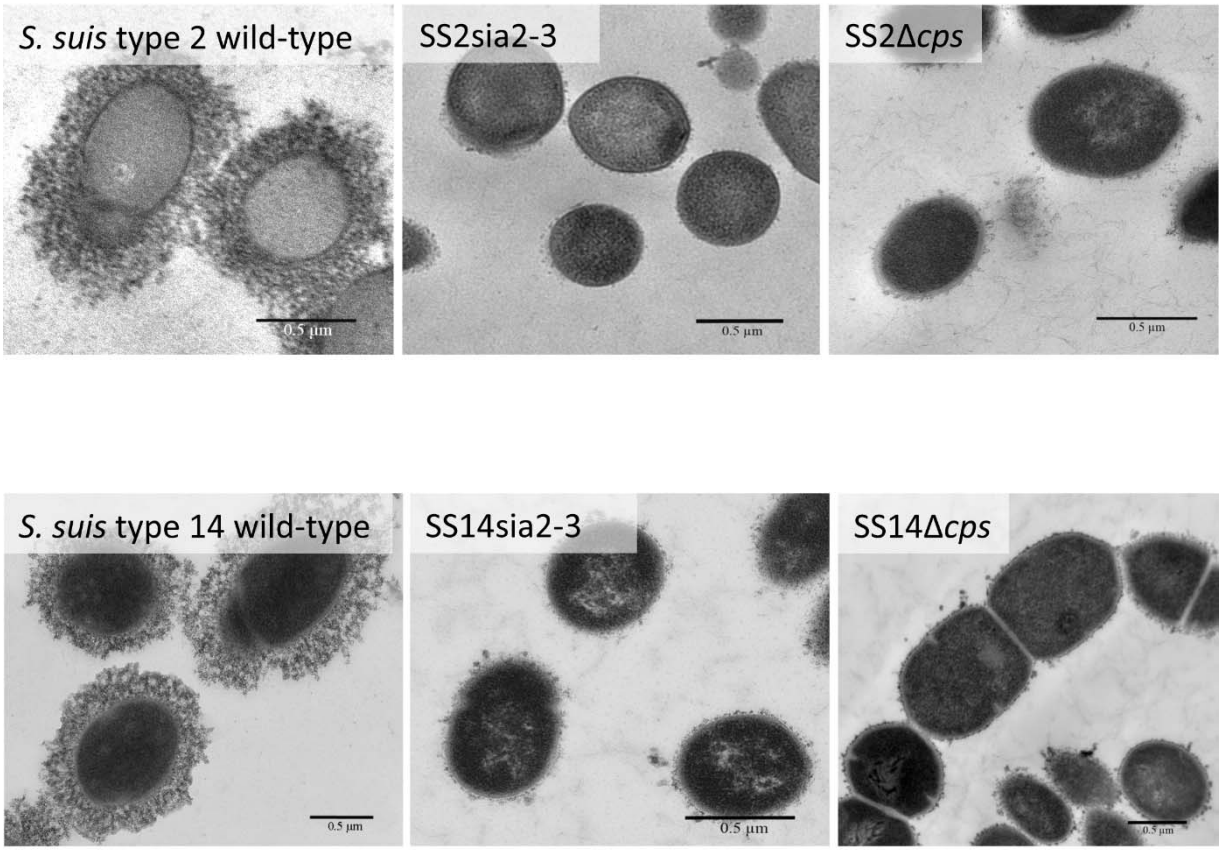


Figure 6. Transmission electron micrographs showing capsular polysaccharide (CPS) expression by *S. suis* serotype 2 and 14 isogenic mutants.

The CPS was labelled with polycationic ferritin. *S. suis* serotype 2 wild-type strain and *S. suis* serotype 14 wild-type strain were surrounded by a thick capsule, whereas the SS2sia2,3 (Δ*cps2N*/*cpsK*) and the SS14sia2,3 (Δ*cps14N*/*cpsK*) were non-encapsulated. Non-encapsulated mutant strains SS2Δ*cps* and SS14Δ*cps* were included as negative controls. Bars = 0.5 µm.

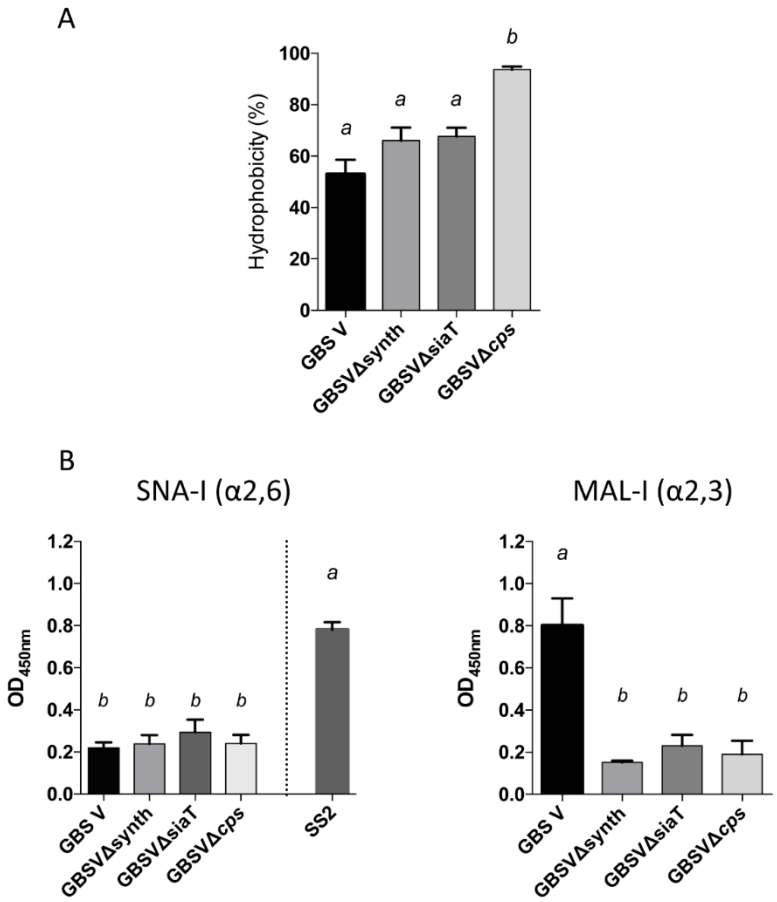


Figure 7. Capsular polysaccharide (CPS) expression levels and recognition of specific CPS sialic acid linkage in GBS type V isogenic mutants.

(A) Hydrophobicity (%) of the wild-type GBS serotype V strain (GBS V), and the sialic acid synthesis GBSVΔsynth (Δ*neu5B*) and sialyltransferase GBSVΔsiaT (Δ*cps5K*) deficient mutants. The non-encapsulated strain (GBSVΔ*cps*) was used as control. (B) Whole-bacterial cell enzyme-linked lectin assay (ELLA) was performed to detect α-2,3 or α-2,6 capsular sialic acid linkage in these mutant strains. Whole bacteria were incubated with *Sambucus nigra* agglutinin (SNA-I) specific for Neu5Ac α-2,6 linkages, or *Maackia amurensis* leukoagglutinin (MAL-I) specific for Neu5Ac α-2,3 linkages. The non-encapsulated mutant GBSVΔ*cps* was used as negative control. *S. suis* serotype 2 (SS2) was used as positive control for SNA-I and wild-type GBS type V as positive control for MAL-I. Data in (A) and (B) are expressed as mean ± SEM of at least three independent experiments. Student *t*-test analyses reported significant differences between '*a*' and '*b*' ($P < 0.05$).

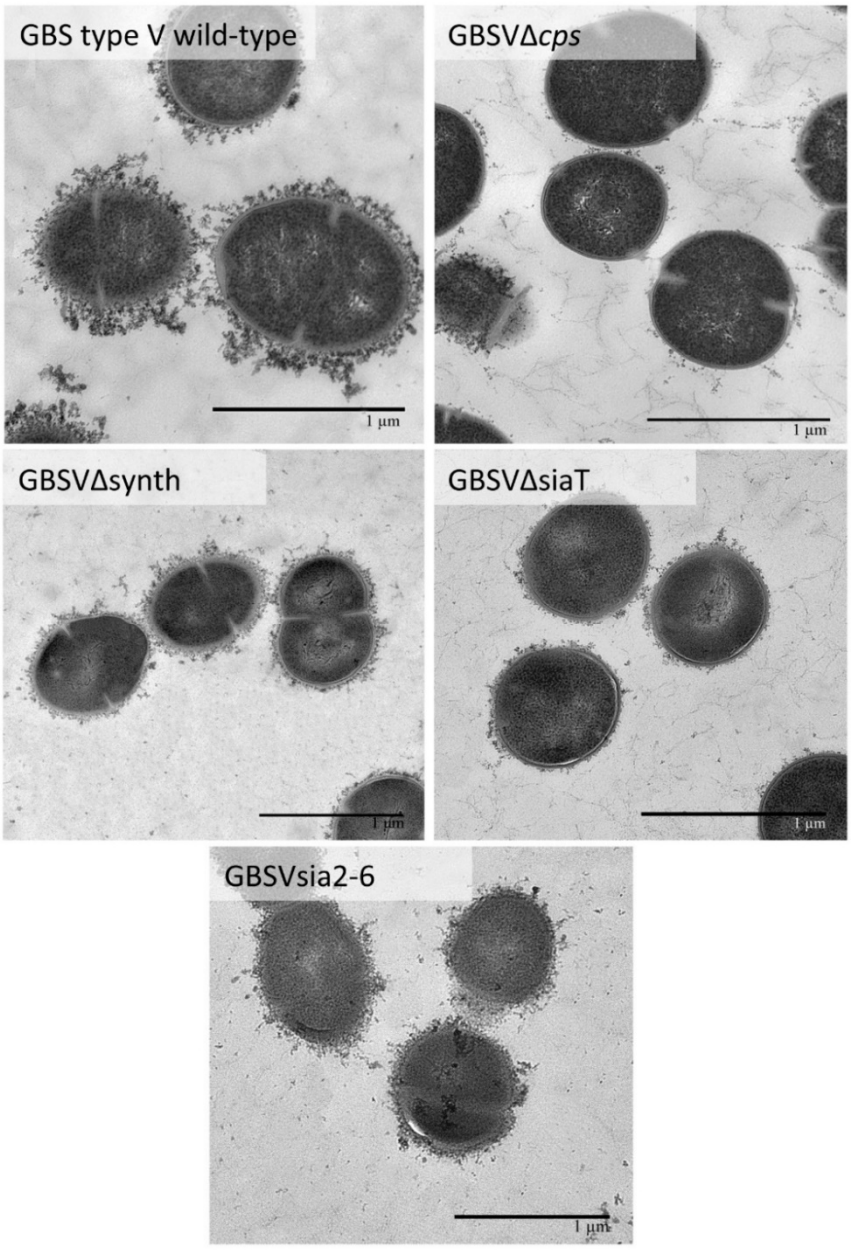


Figure 8. Transmission electron micrographs showing capsular polysaccharide (CPS) expression by GBS type V isogenic mutants.

The CPS was labelled with polycationic ferritin. GBS type V wild-type strain was surrounded by a consistent CPS layer, whereas the GBSVΔsiaT (Δ*cps5K*), the GBSVΔsynth (Δ*neu5B*), and the GBSVsia2,6 (Δ*cps5K*/*cps2N*) mutants all showed intermediate state of encapsulation. The non-encapsulated mutant strain GBSVΔ*cps* is depicted as negative control. Bars = 1 µm.

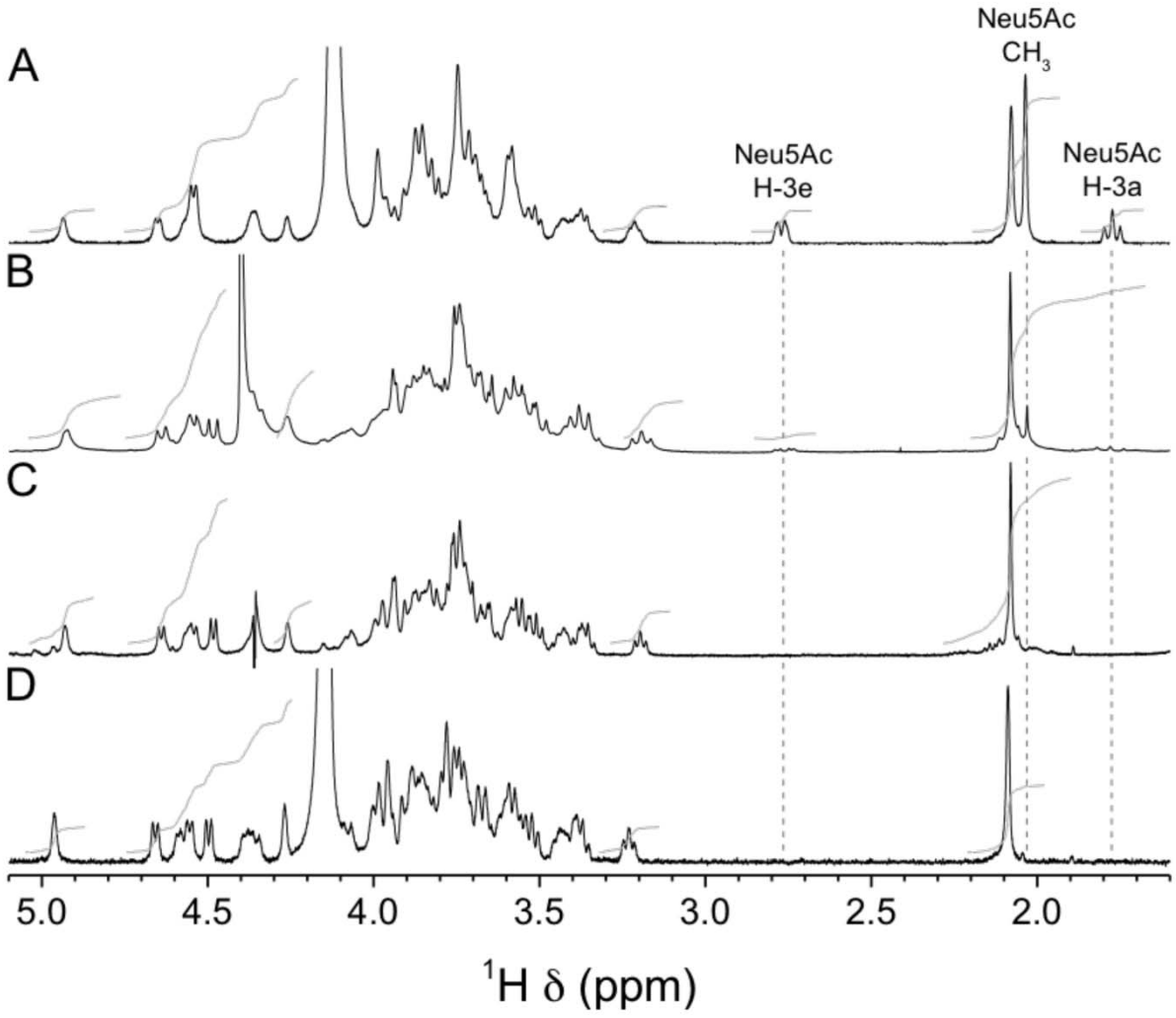


Figure 9. ^{1}H NMR spectra of native GBS type V and sialic acid-deficient mutant capsular polysaccharides (CPSs).

(A) Wild type, native CPS, D_2O, 500 MHz, 30° pulse, 80°C. (B) GBSVΔsynth (Δ*neu5B*) mutant, native CPS, D_2O, 300 MHz, 90° pulse, 60°C. (C) GBSVΔsiaT (Δ*cps5K*) mutant, native CPS, 33 mM phosphate pD 8.0 in D_2O, 500 MHz, 90° pulse with presaturation, 67°C. (D) Wild type, chemically desialylated polysaccharide, D_2O, 500 MHz, 30° pulse, 80°C.

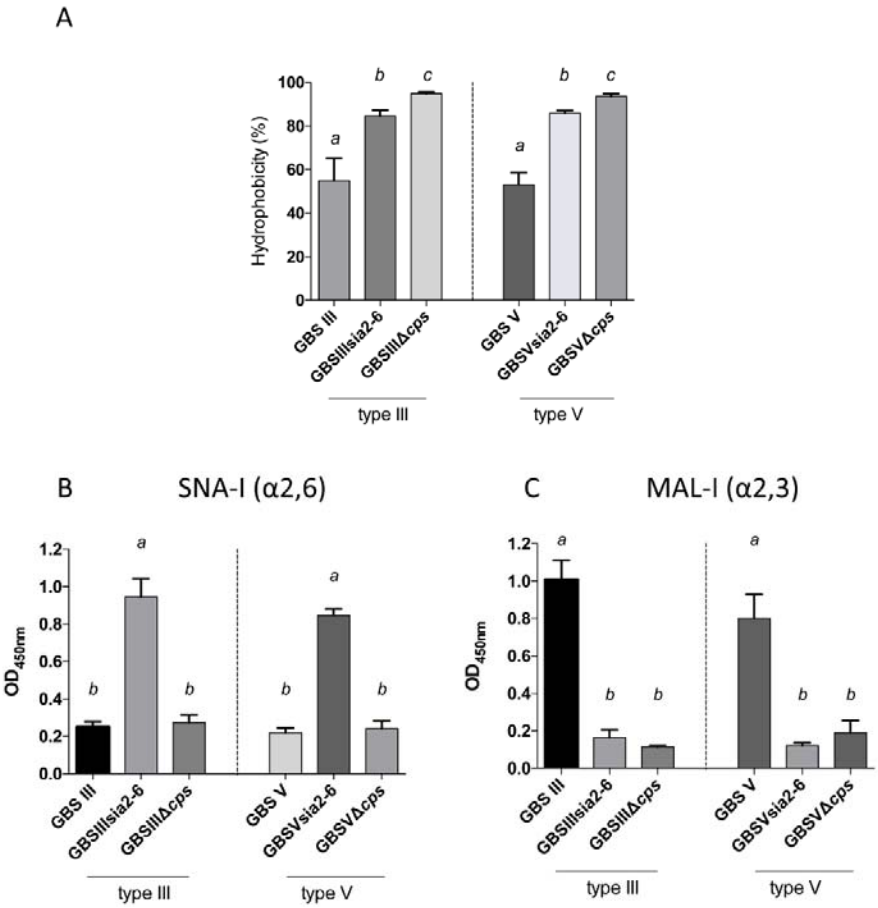


Figure 10. Capsular polysaccharide (CPS) expression levels and sialic acid linkage in GBS types III and type V mutants carrying exogenous α-2,6-sialyltransferase.

(A) Hydrophobicity (%) of GBS type III and V wild-type strains and the mutants GBSIIIsia2,6 (Δ*cps3K*/*cps2N*) and GBSVsia2,6 (Δ*cps5K*/*cps2N*) carrying the *S. suis* α-2,6-sialyltransferase. The non-encapsulated mutant strains (GBSIIIΔ*cps* and GBSVΔ*cps*) were used as controls. (B-C) Whole-bacterial cell enzyme-linked lectin assay (ELLA) was performed to detect α-2,3 or α-2,6 capsular sialic acid linkage in these mutant strains. Whole bacteria were incubated with *Sambucus nigra* agglutinin (SNA-I) specific for Neu5Ac α-2,6 linkages, or *Maackia amurensis* leukoagglutinin (MAL-I) specific for Neu5Ac α-2,3 linkages. The non-encapsulated mutants were used as negative controls. Data in (A), (B), and (C) are expressed as mean ± SEM of at least three independent experiments. Student *t*-test analyses reported significant differences between '*a*′ and '*b*', between '*a*′ and '*c*', and between '*b*′ and '*c*′ ($P < 0.05$).

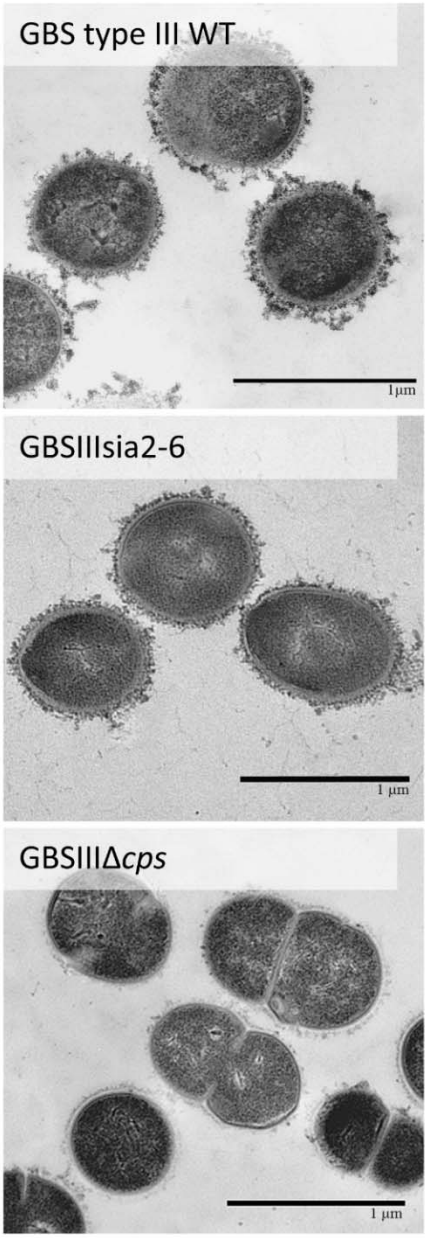


Figure 11. Transmission electron micrographs showing capsular polysaccharide (CPS) expression by GBS type III mutant carrying exogenous α-2,6-sialyltransferase.

The CPS was labelled with polycationic ferritin. GBS type III wild-type strain was surrounded by a consistent CPS layer, whereas the GBSIIIsia2,6 (Δ*cps3K*/*cps2N*) mutant showed intermediate state of encapsulation. The non-encapsulated strain GBSIIIΔ*cps* is depicted as negative control. Bars = 1 µm.

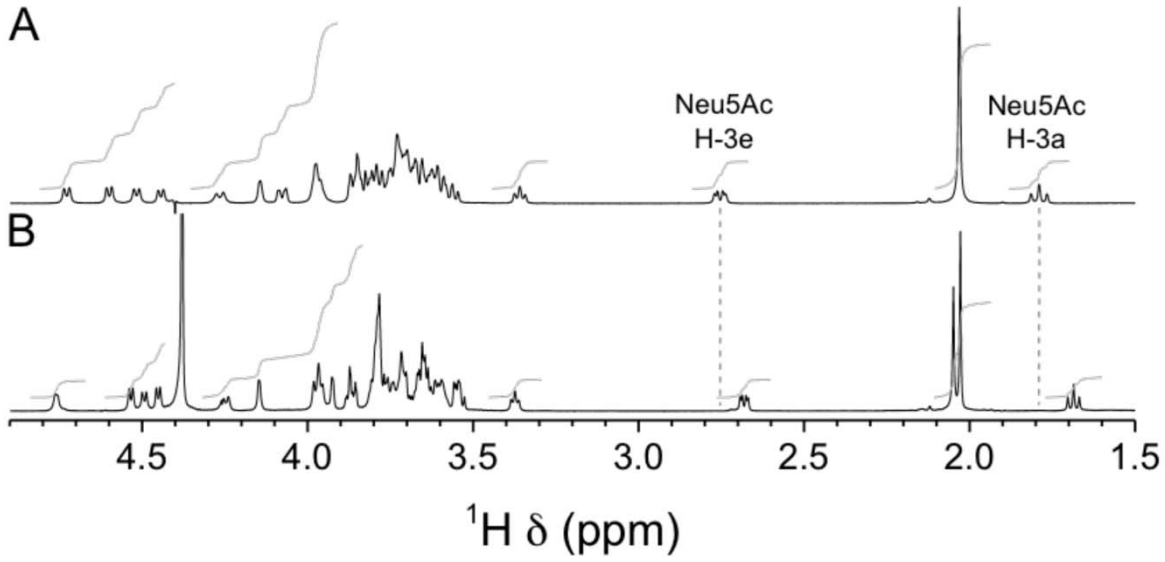


Figure 12. ^{1}H NMR spectra of native GBS type III and mutant GBSIIIsia2,6 capsular polysaccharide (CPSs).

(A) wild type GBS type III, D_2O, 500 MHz, 90° pulse with presaturation, 62°C. (B) GBSIIIsia2,6 (Δ*cps3K*/*cps2N*) mutant, 33 mM phosphate pD 8.0 in D_2O, 700 MHz, 90° pulse, 65°C.

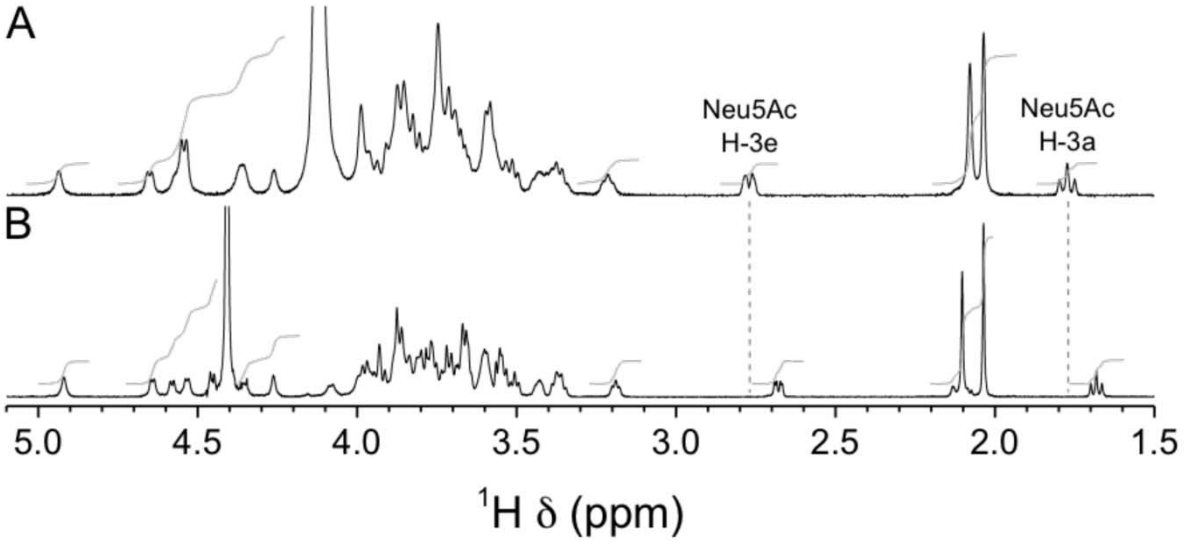


Figure 13. ^{1}H NMR spectra of native GBS type V and mutant GBSVsia2,6 capsular polysaccharides (CPSs).

(A) Wild type GBS type V, D_2O, 500 MHz, 30° pulse, 80°C. (B) GBSVsia2,6 (Δ*cps5K/cps2N*) mutant, 33 mM phosphate pD 8.0 in D_2O, 700 MHz, 30° pulse, 61°C.

Supporting information

Supplementary material is available with the article through the journal Web site at https://www.frontiersin.org/articles/10.3389/fmicb.2018.00545/full#supplementary-material.

Supplementary Table S1. Oligonucleotide primers used in this study.

Supplementary Table S2. ^{1}H and ^{13}C NMR chemical shifts of the GBSIIIsia2,6 mutant CPS.

Supplementary Table S3. ^{1}H and ^{13}C NMR chemical shift differences between the GBSIIIsia2,6 mutant and wild-type GBS type III CPSs.

Supplementary Table S4. ^{1}H and ^{13}C NMR chemical shifts of the GBSVsia2,6 mutant CPS.

Supplementary Figure S1. Portions of the 700-MHz 2D NMR COSY spectrum of the GBSIIIsia2,6 mutant CPS in 33 mM phosphate pD 8.0 in D2O at 65°C.

Supplementary Figure S2. Portions of the 176-MHz ge-2D NMR HSQC spectrum of the GBSIIIsia2,6 mutant CPS in 33 mM phosphate pD 8.0 in D2O at 65°C.

Supplementary Figure S3. Portions of the 700-MHz 2D NMR COSY spectrum of the GBSVsia2,6 mutant CPS in 33 mM phosphate pD 8.0 in D2O at 61°C.

Supplementary Figure S4. Portions of the 176-MHz ge-2D NMR HSQC spectrum of the GBSVsia2,6 mutant CPS in 33 mM phosphate pD 8.0 in D2O at 61°C.

ANNEXES - ARTICLE XIV

Sialylation of *Streptococcus suis* serotype 2 is essential for capsule expression but is not responsible for the main capsular epitope

---

Marie-Pier Lecours [1,$], Nahuel Fittipaldi [2,$], Daisuke Takamatsu [3], Masatoshi Okura [3], Mariela Segura [1], **Guillaume Goyette-Desjardins** [1], Marie-Rose Van Calsteren [4], and Marcelo Gottschalk [1]

[$] These authors equally contributed to this work.

[1] Faculté de médecine vétérinaire, Université de Montréal, 3200 Sicotte, St-Hyacinthe, Quebec J2S 2M2, Canada;
[2] Department of Pathology and Genomic Medicine, The Methodist Hospital System, 6565 Fannin, Houston, TX 77030, USA.
[3] Bacterial and Parasitic Diseases Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan;
[4] Food Research and Development Centre, Agriculture and Agri-Food Canada, 3800 Casavant Ouest, Saint-Hyacinthe, QC J2S 8E3, Canada;

Publié dans *Microbes and Infection*. **2012**. 14(11):641-950.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé activement à la réalisation des expériences (purifications et analyses par lectines; 20%) et à l'analyse des résultats.

Abstract

The capsular polysaccharide is a critical virulence factor of the swine and zoonotic pathogen *Streptococcus suis* serotype 2. The capsule of this bacterium is composed of five different sugars, including terminal sialic acid. To evaluate the role of sialic acid in the pathogenesis of the infection, the *neuC* gene, encoding for an enzyme essential for sialic acid biosynthesis, was inactivated in a highly virulent *S. suis* serotype 2 strain. Using transmission electron microscopy, it was shown that inactivation of *neuC* resulted in loss of expression of the whole capsule. Compared to the parent strain, the Δ*neuC* mutant strain was more phagocytosed by macrophages and was also severely impaired in virulence in a mouse infection model. Both native and desialylated *S. suis* serotype 2 purified capsular polysaccharides were recognized by a polyclonal anti-whole cell *S. suis* serotype 2 serum and a monospecific polyclonal anti-capsule serotype 2 serum. In contrast, only the native capsular polysaccharide was recognized by a monoclonal antibody specific for the sialic acid moiety of the serotype 2 capsule. Together, our results infer that sialylation of *S. suis* serotype 2 may be essential for capsule expression, but that this sugar is not the main epitope of this serotype.

1. Introduction

Streptococcus suis is a major agent of meningitis and septicemia in swine and is also a zoonotic pathogen [1,2]. Although in the past *S. suis* disease in humans was rare and limited to people in close contact with pigs or pork by-products, in recent years *S. suis* has emerged as an important threat to human health, especially in South East and East Asia. This pathogen is now considered as the leading cause of adult meningitis in Vietnam, the second in Thailand and the third in Hong Kong. In the last years, two important outbreaks took place in China with a high fatality rate [1].

Among the 35 *S. suis* serotypes that have been described, serotype 2 is the most virulent for both pigs and humans. The capsular polysaccharide (CPS), which defines the serotype [3], is essential for the virulence of *S. suis* mainly because of its antiphagocytic activity [4]. CPS biosynthesis is driven by enzymatic machinery encoded by genes located in a single, discrete capsular locus (*cps*) [5,6]. Inactivation of genes within this locus, such as *cps2B*, *cpsE* and *cpsF*, resulted in severely decreased (*cpsB*) or completely abrogated capsule production (*cpsEF*) [5–7]. The serotype 2 CPS was recently isolated, purified and characterized [8]. Sugar and absolute configuration analyses of the CPS gave the following composition: D-galactose, D-glucose, D-N-acetylglucosamine, D-Neu5Ac (sialic acid) and L-rhamnose. The CPS structure was also determined. Interestingly, sialic acid was found to be terminal, and the CPS was quantitatively desialylated by mild acid hydrolysis. Consistently with a sialylated CPS, four genes involved in sialic acid biosynthesis (SSU0355, SSU0536, SSU0537 and SSU0538) are found in the *cps* locus of *S. suis*[6,9]. These 4 genes are homologous to, and organized in the same way as, the Group B *Streptococcus* (GBS) sialic acid biosynthesis genes *neuBCDA*. High homologies at the amino acid level between *S. suis* and GBS deduced proteins are observed (Supplemental Table 1). In GBS, homologs of the *neuB* and *neuD* genes have been characterized. They encode a sialyl synthetase and a sialyl O-acetyltransferase, respectively [10]. A homolog of the *neuC* gene has been characterized in *Escherichia coli* K1 as an UDP-N-acetylglucosamine 2-epimerase necessary for sialic acid synthesis [11]. The *neuA* gene was recently described as an O-acetylesterase in *S. suis*[12].

Capsular sialic acid is known to be important for the pathogenesis of other meningitis-causing pathogens, such as *E. coli* K1, *Neisseria meningitidis* and GBS type III [13,14]. In this latter species, sialic acid is also structurally terminal and thought to be a major virulence factor involved, among others, in complement evasion and prevention of phagocytosis [15]. However, the sialic acid linkage to the CPS backbone differs between GBS type III (α-2,3) and *S. suis* serotype 2 (α-2,6), both linked to galactose [8]. Previous GBS mutagenesis studies reported that mutation of *cpsK* gene, coding for a sialyltransferase, resulted in reduced CPS expression [16]. In addition, the effect of sialic acid on CPS immunogenicity of GBS type III is complex, as it exerts conformational control of the dominant antigenic epitope [17].

The role of sialic acid in the pathogenesis of the infection caused by *S. suis* as well as its contribution to the epitope involved in the capsular serotype recognition are poorly known. In the present study, a *S. suis* mutant strain defective for the production of sialic acid was generated and characterized. Results showed that, similarly to GBS type III, such a mutant was also defective in CPS expression at the bacterial surface. In addition, and differently from the latter pathogen, sialic acid in the CPS is not responsible for the immunodominant capsular epitope of *S. suis* serotype 2.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains, derived mutants and plasmids used in this study are listed in Table 1. Wild type (WT) strain P1/7 is a virulent *S. suis* serotype 2 strain whose genome has been sequenced [9]. GBS WT strain COH-1 (kindly provided by Dr. C.E. Rubens, Children's Hospital and Medical Center, University of Washington, Seattle, WA, USA) is a type III highly encapsulated strain used and described in several previous studies [16,25,26]. Streptococcal strains were grown on Todd-Hewitt broth (THB) or agar (THA) (Becton Dickinson, Mississauga, ON, Canada) or on sheep blood agar plates at 37 °C. *E. coli* strains were grown on Luria-Bertani broth or agar (Becton Dickinson). When needed, antibiotics (Sigma, Oakville, ON, Canada) were added to the culture media at the following concentrations: for E. coli, kanamycin (Km) and spectinomycin (Sp) were added at 50 μg/ml;

for *S. suis* and GBS, Sp was added at 100 µg/ml and 200 µg/ml, respectively. For comparative purposes, a previously characterized CPS-deficient mutant strain derived from *S. suis* P1/7 strain, obtained by precise in-frame deletion of *cpsF* gene coding for CPS biosynthesis, was also included [7]. To perform *S. suis*-macrophage interaction studies, isolated colonies were used as inocula for THB, which was incubated 16 h at 37 °C with agitation. Working cultures for cell stimulation were obtained by inoculating 300 µl of the 16 h-culture into 10 ml of THB that were incubated for 5 h at 37 °C with shaking. Bacteria were washed twice in phosphate-buffered saline (PBS), pH 7.3, and appropriately diluted in complete cell culture medium consisting of DMEM (Gibco, Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) for the experiments. The number of CFU/ml in the final suspension was determined by plating samples onto THB agar using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA, USA). For in vivo infections, isolated colonies were used as inocula for THB, which was incubated 8 h at 37 °C with agitation. Working cultures were obtained by inoculating 10 µl of a 10−3 dilution of these cultures in 30 ml of THB and incubating for 16 h at 37 °C with agitation. Bacteria were washed twice in PBS and were appropriately diluted in THB for the inoculation. To perform dot-ELISA, bacteria were grown overnight in 10 ml of THB, washed with PBS and resuspended in 1 ml of PBS-0.5% formalin (whole bacteria with a concentration equivalent to 109 bacteria/ml).

2.2. DNA manipulations

S. suis genomic DNA was prepared by the guanidium thiocyanate method [27]. Minipreparations of recombinant plasmids and transformation of *E. coli* were performed by standard procedures [28]. Restriction enzymes and DNA-modifying enzymes were purchased from TaKaRa Bio (Otsu, Shiga, Japan) and used according to the 'manufacturers' recommendation. PCR reactions were carried out with the iProof proofreading DNA polymerase (BioRad Laboratories, Mississauga, ON, Canada). Oligonucleotide primers were from Invitrogen (Burlington, ON, Canada). Amplification products were purified on Sephadex S-400 columns (GE Healthcare, Mississauga, ON, Canada) and sequenced with an ABI 3730xl automated DNA sequencer, using the ABI PRISM dye terminator cycle version 3.1 (Applied Biosystems, Carlsbad, CA, USA).

2.3. Construction of the S. suis and GBS mutants

In the case of *S. suis*, the *neuC* gene was targeted, since it codes for a protein involved in sialic acid synthesis [29]. For comparative purposes, GBS type III mutants defective in either *cpsE* (1032 bp, gene involved in CPS biosynthesis) or *neuB* (894 bp, gene involved in sialic acid synthesis) were also generated. The DNA genome sequences of *S. suis* serotype 2 strain P1/7 and GBS strain COH-1 were retrieved from GenBank (Accession Numbers AM946016 and AF16383, respectively). In-frame deletions of target genes were constructed by using splicing-by-overlap-extension PCR [30] and the primers listed in Table 2. The PCR-generated deletion alleles were cloned into plasmid pCR2.1 (Invitrogen), extracted with BamHI and PstI and recloned into the thermosensitive *E. coli–S. suis* shuttle plasmid pSET4s [23] digested with the same enzymes, giving rise to the knockout vectors p4Δ*neuC* for *S. suis* and p4Δ*cpsE* and p4Δ*neuB* for GBS. Electroporation of *S. suis* strain P1/7 and GBS strain COH-1 and procedures for isolation of mutants were those described previously [31]. Allelic replacement was confirmed by PCR and DNA sequencing analysis. Serotyping of *S. suis* and GBS mutants was performed by coagglutination test by using either polyclonal hyperimmune serum as described for *S. suis*[32] or commercial rabbit antisera against GBS type III capsular material (Denka Seiken, Campbell, CA) [33].

For complementation, the full length *neuC* gene was amplified from genomic DNA of *S. suis* P1/7 using primers described in Table 2, digested with PstI, and cloned into pMX1 via the same restriction site. Plasmid pMX1 is a derivative of the *S. suis–E. coli* shuttle cloning vector pSET2 and possesses the *S. suis malX* promoter for transgene expression in *S. suis* [23]. The resulting complementation vector pCompNeuC was introduced into the Δ*neuC* mutant by electroporation. The presence of CPS was studied for both pathogens and their mutants by electron microscopy, as described below.

2.4. Transmission electron microscopy

The presence of capsule in the mutant derivatives was verified by transmission electron microscopy (TEM). Bacterial cells (*S. suis* and GBS) were fixed for 2 h at 20 °C in cacodylate buffer containing 5% glutaraldehyde. Fixed bacteria were suspended in cacodylate buffer and allowed to react with polycationic ferritin for 30 min at 20 °C. The reaction was slowed by 10-

fold dilution with buffer, and the organisms were centrifuged and washed three times in cacodylate buffer. Bacterial cells were then immobilized in 4% agar, washed 5 times in cacodylate buffer, and postfixed with 2% osmium tetroxide for 2 h. Washing steps were repeated as above, and the samples were dehydrated in a graded series of ethanol washes. All solutions used in processing the specimens contained 0.05% (wt/vol) ruthenium red. Samples were then washed twice in propylene oxide and embedded with LR White resin. Thin sections were poststained with uranyl acetate and lead citrate and examined with an electron microscope JEOL JEM-1230 (Tokyo, Japan).

2.5. S. suis phagocytosis assay

J774-A1 macrophages (ATCC TIB 67, Rockville, MD, USA.) were used for *S. suis* phagocytosis studies as previously described [26]. Macrophages (10^5 cells/ml) were infected with *S. suis* strains (WT, Δ*neuC* mutant and complemented Δ*neuC* (Δ*neuC*/*neuC*) at a concentration of 1×10^7 CFU/ml (MOI: 100) for 90 min at 37 °C with 5% CO_2. Assay conditions were chosen based on preliminary studies and previous data on the kinetics of *S. suis* phagocytosis by J774-A1 macrophages ([26] and data not shown). After incubation, penicillin G (5 μg/ml) and gentamycin (100 μg/ml) (both from Sigma) were directly added into the wells for 1 h to kill extracellular bacteria. Supernatant controls were taken in every test to confirm that extracellular bacteria were efficiently killed by the antibiotics. After antibiotic treatment, cells were washed 3 times, and sterile water was added to lyse the cells. To ensure complete cell lysis, cells were disrupted by scraping the bottom of the well and by vigorous pipetting. All samples were plated using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech). Each test was repeated at least four times in independent experiments, and the number of CFU recovered per well (mean number ± SEM) was determined.

2.6. S. suis in vivo infection

A well-standardized model of murine infection was used [34]. Female, 6-week-old CD1 mice (Charles River Laboratories, Wilmington, MA, USA) were acclimated to standard laboratory conditions of 12 h light/12 h dark cycle with free access to rodent chow and water. All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care

and the Use of Laboratory Animals by the Animal Welfare Committee of Université de Montréal. A total of 35 animals, divided in 2 groups of 15 animals (P1/7 and ΔneuC) and 1 group of 5 animals (non-infected control) were included in the study. On the day of the experiment, a 1-ml volume of the bacterial suspension (5×10^7 CFU/ml) or the vehicle solution (sterile THB) was administrated by i.p. injection. Mice were monitored at least three times daily for mortality and clinical signs of septic disease, such as depression, swollen eyes, rough hair coat, lethargy, and nervous signs of meningitis. Blood samples were collected from the tail at 24, 48 and 72 h post-infection (pi), and plated onto blood agar plates. Blood agar plates were incubated overnight at 37 °C. Colonies were counted as described above and expressed as CFU/ml of blood.

2.7. S. suis CPS purification and CPS desialylation

S. suis serotype 2 reference strain S735 was grown in 50 ml of THB at 37 °C for 18 h, diluted to 2 l in fresh THB, and grown to an OD540 of 0.8. The cells were pelleted by centrifugation at 10 000× g for 40 min, suspended in 33 mmol/l PBS pH 8.0 by repeated pipetting, and chilled. The CPS was prepared and purified as previously described [8]. For quality controls, CPS was analyzed by nuclear magnetic resonance (NMR). Lack of protein and RNA/DNA contamination was verified by Lowry method and by spectrophotometry, respectively. When needed, CPS was desialylated by mild acid hydrolysis. CPS (8 mg) was heated in 1 ml of 70 mmol/l HCl at 60 °C for 4 h, neutralized with 2 mol/l NH4OH, and purified on a Sephadex G10 column (1.5 × 10 cm) (GE Healthcare). Presence (native CPS) or absence (desialylated CPS) of sialic acid was verified by gas chromatography after methanolysis and acetylation and by NMR, and by a reaction with an enzyme linked lectin assay (ELLA) as described below.

2.8. Enzyme linked lectin assay (ELLA) to detect the presence of sialic acid

In order to verify the presence or absence of sialic acid in the purified native and desialylated CPS, an ELLA test was carried out with the Sambucus nigra lectin (SNA-I, Vector Labs Canada, Burlington, ON, Canada), which specifically recognizes sialic acid as α-Neu5Ac-2,6-D-Galp/GalpNAc [35]. The test was based on a previous described technique [36], adapted to the CPS. Briefly, 200 ng of sample (native CPS, desialylated CPS or skimmed

milk as positive control) were added to wells of an ELISA plate (Nunc-Immuno Polysorp, Canadawide Scientific, Toronto, ON, Canada). After being coated, wells were washed and blocked (to avoid non-specific binding) by the addition of Carbo-Free solution 1X (Vector Labs). After washings, biotinylated SNA-I, followed by horseradish peroxidase-labeled Avidin D (Vector Labs) and 3,3′,5,5′-tetramethylbenzidine were added. The enzyme reaction was stopped with the addition of 1N H2SO4 and the absorbance was read at 450 nm with an ELISA plate reader.

2.9. Dot-ELISA

Ten µl of native or desialylated purified *S. suis* serotype 2 CPS (each at 1 mg/ml), or 10 µl of a formalin-killed whole-bacteria suspension were blotted on a PVDF Western blotting membrane (Roche, Mississauga, ON, Canada). The membrane was blocked for 1 h with a solution of Tris-buffered saline (TBS) containing 2% casein, followed by a 2-h incubation with either monoclonal antibody Z3 supernatant [37], rabbit serum (anti-*S. suis* 735) [38] or adsorbed rabbit serum (polyclonal anti-*S. suis* capsule, see below). A second polyclonal antibody, produced against the P1/7 strain, was also used. The membrane was washed and appropriate rabbit or mice conjugated antibody was added for 1 h. The membrane was rinsed 3 times with TBS and revealed with a 4-chloro-1-naphthol solution.

Hyperimmune serum was adsorbed with a non-encapsulated mutant (BD101) derived from strain S735 [21] to selectively obtain antibodies against the CPS. Briefly, an overnight culture of *S. suis* strain BD101 was pelleted and resuspended with 10 ml of PBS. The sample was then divided into two 5-ml aliquots, and centrifuged again. One of the two pellets was resuspended in 3 ml of polyclonal serum anti-*S. suis* strain S735, and incubated for 2 h with agitation. The second pellet was kept on ice for the next adsorption. After 2 h, the first suspension was centrifuged, and the supernatant transferred to the second bacterial pellet. After 2 h of incubation, the suspension was centrifuged, and the supernatant recovered and filtered (0.22 µm). A negative reaction with the BD101 mutant, but not with the WT strain S735 was confirmed by dot-ELISA (results not shown). Similarly, antiserum against strain P1/7 was produced and adsorbed with ΔcpsF strain as described above.

2.10. Statistical analysis

All data are expressed as mean ± SE. For all experiments, except in vivo virulence, data were analyzed for significance using Student's unpaired t-test. For in vivo virulence experiments, survival was analyzed with the LogRank test. A P value < 0.05 was used as a threshold for significance. Independent experiments were repeated at least three times.

3. Results

3.1. Inactivation of neuC in S. suis serotype 2 results in absence of CPS

To verify whether the *S. suis* Δ*neuC* mutant strain was still typable, it was tested by the coagglutination test. Interestingly, both Δ*cpsF* and Δ*neuC* mutants showed absence of reaction with an anti-serotype 2 serum (data not shown). To verify if this negative result was the consequence of the absence of capsular material in the sialic acid negative mutant, both mutants were also tested by TEM. Results showed similar absence of polycationic ferritin marker in both of them, indicating absence of CPS (Fig. 1B and C), when compared to the WT strain (Fig. 1A). The Δ*neuC*/*neuC* strain showed an intermediate expression of CPS, showing that complementation partially restores the phenotype (Fig. 1D).

As previously described for GBS type III Δ*cpsK* and Δ*neuA* mutants [16,39], an important reduction of CPS at the cell surface was observed with the GBS Δ*neuB* mutant obtained in this study (Fig. 1G) compared to the WT strain (Fig. 1E); on the other hand, a complete absence of CPS was observed in the GBS mutant defective in CPS biosynthesis (*cpsE*) (Fig. 1F). As expected, results from the coagglutination test revealed a clear positive reaction with the WT strain, a weak but still positive reaction with the Δ*neuB* mutant and a negative reaction with the Δ*cpsE* non-encapsulated mutant.

3.2. The ΔneuC mutant is highly phagocytosed by macrophages

To determine the ability of J774 macrophages to internalize *S. suis*, bacteria were incubated with cells for 90 min. As shown in Fig. 2A, the WT strain P1/7 was relatively resistant to phagocytosis and relatively few bacteria were found inside the cells. On the other hand, the Δ*neuC* mutant strain was significantly (P < 0.05) more internalized by macrophages.

The Δ*neuC*/*neuC* strain was significantly less internalized by macrophages than the Δ*neuC* strain, but still more phagocytosed than the WT strain ($P < 0.05$), indicating that the level of phagocytosis would be related to the amount of CPS at the bacterial surface, as showed by TEM.

3.3. In vivo infection

Most mice in the WT group presented severe clinical signs associated with septicemia, such as depression, swollen eyes, rough hair coat, prostration and weakness during the first 72 h pi. Twenty-seven percent of animals died or were euthanized for ethical reasons within 72 h (Fig. 2B). *S. suis* could be isolated from blood samples of the WT infected mice within the first 72 h. Still in the WT group, remaining 20% of the animals died or were euthanized for ethical reasons between 3 days and 7 days pi, after the development of nervous signs associated to meningitis. In the Δ*neuC* mutant group, animals did not present any clinical signs associated with septicemia or meningitis, and *S. suis* could not be isolated from blood samples (data not shown).

3.4. Sialylation of CPS is not responsible for the conformational immunodominant epitope of S. suis serotype 2 capsular polysaccharide

Sialic acid may play a critical role as part of a major conformational epitope of bacterial CPS, as it is the case for GBS type III. On other cases, such as GBS type V, capsular sialic acid is dispensable for the main epitope of this serotype [17–40]. In this study, the role of the capsular sialic acid in the conformational epitope of *S. suis* was studied. Purified native and desialylated *S. suis* serotype 2 CPS were dotted on a membrane and incubated with either a monoclonal antibody against the sialic acid moiety or a polyclonal serum against whole bacteria. As shown in Fig. 3A, the monoclonal antibody recognized the native CPS and whole bacteria, but not the desialylated CPS, as expected. To demonstrate that the monoclonal antibody recognizes an epitope containing the sialic acid moiety, an SNA-I ELLA showed that native CPS, but not desialylated CPS, was clearly recognized by the lectin (Fig. 4). Interestingly, the polyclonal serum recognized native and desialylated CPS as well as whole bacteria (Fig. 3B), indicating that the sialic acid moiety is not essential for CPS recognition by

a serotype-specific antibody. Identical results were obtained with monospecific polyclonal antibodies against the CPS of strain S735 (Fig. 3C) or strain P1/7 (results not shown).

4. Discussion

S. suis is a major swine pathogen and a zoonotic agent of increasing importance [1]. The CPS is considered a critical *S. suis* virulence factor that protects bacteria from killing in the bloodstream. The CPS was previously shown to be sialylated [8]. Capsular sialic acid is a well-known virulence factor for several bacteria causing meningitis. For example, it has been proposed that sialylation of GBS CPS is critical for prevention of opsonophagocytosis through inhibition of alternative complement pathway activation [39]. In the case of *S. suis*, very little is known concerning the role of its capsular sialic acid. Charland et al. reported that no differences could be found in sialic acid concentrations between strains of different virulence degrees [41]. In addition, no significant differences could be found in the phagocytosis rate by porcine blood monocytes of *S. suis* treated or not with sialidase or SNA-I [41]. These preliminary results suggested that sialic acid itself might not play an important role in *S. suis* virulence. Although limited knowledge is available concerning the genes involved in sialic acid production in *S. suis*, putative roles can be inferred from other organisms [10–12]. In order to further study the role of capsular sialic acid in the pathogenesis of *S. suis* infection, we obtained a nonpolar mutant defective in the production of sialic acid, targeting the *neuC* gene. Based on previous reports [42], the sialic acid biosynthesis pathway begins with NeuC, which present high homology with NeuC from GBS, as it is the case for NeuB and NeuD (Supplemental Table 1) [29].

A major finding of this work is the observation that Δ*neuC* had an important impact on CPS expression. First, the mutant strain was untypable with current antisera used in serotyping. However, this result could also be the consequence of the influence of sialic acid on the immunodominant capsular epitope for *S. suis* serotype 2. However, TEM analysis showed the importance of *neuC* for CPS expression. The WT strain was surrounded by a thick CPS linked to the polycationic ferritin whereas the Δ*neuC* mutant strain showed absence of CPS, similarly to the non-encapsulated Δ*cpsF* mutant. Complementation partially restored the

phenotype as an intermediate expression of CPS was noticeable with the complemented Δ*neuC*/*neuC* strain. Results obtained imply that loss of sialylation might be related to the loss of CPS at the bacterial surface in *S. suis*. A *neuB* mutant of *S. suis* had been previously obtained by transposon mutagenesis and shown to be non-virulent in pigs [43]. Interestingly, we observed that this mutant was untypable with antiserum against *S. suis* serotype 2 and non-encapsulated by electron microscopy (unpublished observations).

S. suis is frequently compared to GBS type III, both being meningitis pathogens and the only Streptococcus harboring a sialic acid-rich CPS. However, these pathogens possess different linkages of sialic acid to the respective backbone structures [8]. A previous GBS mutagenesis study reported that mutation of *cpsK* gene, coding for a sialyltransferase, resulted in reduced (80%) CPS expression [16]. It was suggested that the sialyltransferase is an integral part of the glycosyltransferase complex, so its loss may also disrupt the functional integrity of whole CPS synthesis complex [16]. We confirmed, for the first time by TEM, that residual capsular material is still present in GBS Δ*neuB* mutant, defective in the biosynthesis of sialic acid, when compared to GBS Δ*cpsE* mutant, which is completely non-encapsulated. These results were confirmed by a weak reaction of the Δ*neuB* mutant by coagglutination with anti-type III antibodies. The lack of sialic acid seems to differently affect the production of the backbone structure depending on the pathogen, being critical for *S. suis* CPS (this study), partially involved in GBS CPS ([16] and this study) and totally dispensable for *Haemophilus ducreyi* and *N. meningitidis*[44].

In the case of GBS type III, the effect of sialic acid on CPS immunogenicity is complex. For example, sialic acid exerts a conformational control of the dominant antigenic epitope in type III but not in type V [17,40]. It had been previously suggested that sialic acid residues in *S. suis* type 2 CPS would be part of the antigen epitope portion of the capsule [45]. However, analysis carried out in this study clearly showed that removal of sialic acid results in loss of reactivity against not only a monoclonal antibody directed to this residue [37] but also a specific lectin [41]. However, it did not prevent the recognition with either a polyclonal antibody routinely used for serotyping or an adsorbed monospecific antibody against the complete CPS. These results indicate that sialic acid in *S. suis* type 2 CPS is not the major or

unique determinant of the serotype. Whether a complete CPS would induce different level and isotypes of antibodies than a desialylated CPS, as it is the case of GBS type V [40], is still unknown and is presently being studied in our laboratory.

Finally, and as expected, the Δ*neuC* behaved as a non-encapsulated mutant being highly susceptible to phagocytosis and non-virulent in a mouse model of infection [26,34]. Caution should be used in evaluating the effect of mutations in sialic acid synthesis and its presence on the CPS regarding phagocytosis and/or virulence, since their effects are confounded with an overall decrease in capsule production. Dissection of the molecular pathways underlying sialic acid-control of *S. suis* CPS expression would let the construction of strains that produce normal levels of asialo-CPS, allowing direct evaluation of sialic acid contribution to bacteria–host interactions.

Acknowledgements

We would like to thank Fleur Gagnon and Sonia Lacouture for their invaluable technical help. This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) grant #154280 as well as Discovery Accelerator Supplement #380299 to MG. M-PL is the recipient of a NSERC doctoral award.

References

[1] M. Gottschalk, J. Xu, C. Calzas, M. Segura. Streptococcus suis: a new emerging or an old neglected zoonotic pathogen? Future Microbiol., 5 (2010), pp. 371-391

[2] M. Gottschalk, M. Segura. The pathogenesis of the meningitis caused by Streptococcus suis: the unresolved questions. Vet. Microbiol, 76 (2000), pp. 259-272

[3] M. Gottschalk. Streptococcosis. in: L. Karriker, A. Ramirez, K. Schwartz, G. Stevenson, J. Zimmerman (Eds.), Diseases of Swine, tenth ed., Wiley Publishers, Hoboken, NJ, 2012, In press.

[4] N. Fittipaldi, M. Segura, D. Grenier, M. Gottschalk. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent Streptococcus suis. Future Microbiol., 7 (2011), pp. 259-279

[5] H.E. Smith, M. Damman, J. van der Velde, F. Wagenaar, H.J. Wisselink, N. Stockhofe-Zurwieden, M.A. Smits. Identification and characterization of the cps locus of Streptococcus suis serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun., 67 (1999), pp. 1750-1756

[6] H.E. Smith, R. de Vries, R. van't Slot, M.A. Smits. The cps locus of Streptococcus suis serotype 2: genetic determinant for the synthesis of sialic acid. Microb. Pathog., 29 (2000), pp. 127-134

[7] M.P. Lecours, M. Gottschalk, M. Houde, P. Lemire, N. Fittipaldi, M. Segura. Critical role for Streptococcus suis cell wall modifications and suilysin in resistance to complement-dependent killing by dendritic cells. J. Infect. Dis., 204 (2011), pp. 919-929

[8] M.R. Van Calsteren, F. Gagnon, S. Lacouture, N. Fittipaldi, M. Gottschalk. Structure determination of Streptococcus suis serotype 2 capsular polysaccharide
Biochem. Cell. Biol., 88 (2010), pp. 513-525

[9] M.T. Holden, H. Hauser, M. Sanders, T.H. Ngo, I. Cherevach, A. Cronin, I. Goodhead, K. Mungall, M.A. Quail, C. Price, E. Rabbinowitsch, S. Sharp, N.J. Croucher, T.B. Chieu, N.T. Mai, T.S. Diep, N.T. Chinh, M. Kehoe, J.A. Leigh, P.N. Ward, C.G. Dowson, A.M. Whatmore, N. Chanter, P. Iversen, M. Gottschalk, J.D. Slater, H.E. Smith, B.G. Spratt, J. Xu, C. Ye, S. Bentley, B.G. Barrell, C. Schultsz, D.J. Maskell, J. Parkhill. Rapid evolution of virulence and drug resistance in the emerging zoonotic pathogen Streptococcus suis. PLoS One (2009)
e6072

[10] M.F. Lartique, A. Poulard Fribourg, R. Al Safadi, A.S. Valentin-Domelier, N. Van Der Mee-Marquet, A. Rosenau, R. Quentin. Variability of neuD transcription levels and capsular sialic acid expression among serotype III Group B Streptococcus strains. Microbiology, 157 (2011), pp. 3282-3291

[11] W.F. Vann, D.A. Daines, A.S. Murkin, M.E. Tanner, D.O. Chaffin, C.E. Rubens, J. Vionnet, R.P. Silver. The NeuC protein of Escherichia coli K1 is a UDP N-acetylglucosamine 2-epimerase. J. Bacteriol., 186 (2004), pp. 706-712

[12] L. Song, H. Zhou, X. Cai, C. Li, J. Liang, C. Jin. NeuA O-acetylesterase activity is specific for CMP-activated O-acetyl sialic acid in Streptococcus suis serotype 2. Biochem. Biophys. Res. Commun., 410 (2011), pp. 212-217

[13] M.R. Wessels, C.E. Rubens, V.J. Benedi, D.L. Kasper. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc. Natl. Acad. Sci. USA., 86 (1989), pp. 8983-8987

[14] S.H. Huang, M.F. Stins, K.S. Kim. Bacterial penetration across the blood-brain barrier during the development of neonatal meningitis. Microbes Infect., 2 (2000), pp. 1237-1244

[15] H.C. Maisey, K.S. Doran, V. Nizet. Recent advances in understanding the molecular basis of group B Streptococcus virulence. Expert Rev. Mol. Med., 10 (2008) e27

[16] D.O. Chaffin, L.M. Mentele, C.E. Rubens. Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. J. Bacteriol., 187 (2005), pp. 4615-4626

[17] H.J. Jennings, C. Lugowski, D.L. Kasper. Conformational aspects critical to the immunospecificity of the type III group B streptococcal polysaccharide. Biochemistry, 20 (1981), pp. 4511-4518

[18] M.J. Casabadan, S.N. Cohen. Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. Mol. Biol., 138 (1980), pp. 179-207

[19] J.D. Slater, A.G. Allen, J.P. May, S. Bolitho, H. Lindsay, D.J. Maskell. Mutagenesis of Streptococcus equi and Streptococcus suis by transposon Tn917. Vet. Microbiol., 29 (2003), pp. 197-206

[20] M. Jacques, M. Gottschalk, B. Foiry, R. Higgins. Ultrastructural study of surface components of Streptococcus suis. J. Bacteriol., 172 (1990), pp. 2833-2838

[21] N. Fittipaldi, J. Harel, B. D'Amours, S. Lacouture, M. Kobisch, M. Gottschalk. Potential use of an unencapsulated and aromatic amino acid-auxotrophic Streptococcus suis mutant as a live attenuated vaccine in swine. Vaccine, 25 (2007), pp. 3524-3535

[22] C.E. Rubens, J.M. Kupers, L.M. Heggen, D.L. Kasper, M.R. Wessel. Molecular analysis of the group B streptococcal capsule genes. G.M. Dunny, P.P. Cleary, L.L. McKay (Eds.), Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci, ASM Press, Washington, DC (1991), pp. pp.179-pp.183

[23] D. Takamatsu, M. Osaki, T. Sekizaki. Thermosensitive suicide vectors for gene replacement in Streptococcus suis. Plasmid, 46 (2001), pp. 140-148

[24] M. Okura, M. Osaki, N. Fittipaldi, M. Gottschalk, T. Sekizaki, D. Takamatsu. The minor pilin subunit Sgp2 is necessary for assembly of the pilus encoded by the srtG cluster of Streptococcus suis. J. Bacteriol., 193 (2011), pp. 822-831

[25] G. Mancuso, M. Gambuzza, A. Midiri, C. Biondo, S. Papasergi, S. Akira, G. Teti, C. Beninati. Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat. Immunol., 10 (2009), pp. 587-594

[26] M.A. Segura, P. Cleroux, M. Gottschalk. Streptococcus suis and Group B Streptococcus differ in their interactions with murine macrophages. FEMS Immunol. Med. Microbiol., 21 (1998), pp. 189-195

[27] D.G. Pitcher, N.A. Saunders, R.J. Owen. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol., 8 (1989), pp. 151-156

[28] J. Sambrook, E.F. Fritsch, T. Maniatis. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory Press, New York (1989)

[29] P.W. Annunziato, L.F. Wright, W.F. Vann, R.P. Silver. Nucleotide sequence and genetic analysis of the neuD and neuB genes in region 2 of the polysialic acid gene cluster of Escherichia coli K1. J. Bacteriol., 177 (1995), pp. 312-319

[30] A.N. Warrens, M.D. Jones, R.I. Lechler. Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest. Gene, 186 (1997), pp. 29-35

[31] D. Takamatsu, M. Osaki, T. Sekizaki. Construction and characterization of Streptococcus suis–Escherichia coli shuttle cloning vectors. Plasmid, 45 (2001), pp. 101-113

[32] M. Gottschalk, R. Higgins, M. Boudreau. Use of polyvalent coagglutination reagents for serotyping of Streptococcus suis. J. Clin. Microbiol., 31 (1993), pp. 2192-2194

[33] D.S. Leland, R.C. Lachapelle, F.M. Wlodarski. Method for rapid detection of group B streptococci by coagglutination. J. Clin. Microbiol., 7 (1978), pp. 323-326

[34] M.C. Dominguez-Punaro, M. Segura, M.M. Plante, S. Lacouture, S. Rivest, M. Gottschalk. Streptococcus suis serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection. J. Immunol., 179 (2007), pp. 1842-1854

[35] N. Shibuya, I.J. Goldstein, W.F. Broekaert, M. Nsimba-Lubaki, B. Peeters, W.J. Peumans. The elderberry (Sambucus nigra L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. J. Biol. Chem., 262 (1987), pp. 1596-1601

[36] O. Gornik, G. Lauc. Enzyme linked lectin assay (ELLA) for direct analysis of transferrin sialylation in serum samples. Clin. Biochem., 40 (2007), pp. 718-723

[37] N. Charland, M. Jacques, S. Lacouture, M. Gottschalk. Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by Streptococcus suis serotypes 1, 2 and 1/2. Microbiology, 143 (1997), pp. 3607-3614

[38] R. Higgins, M. Gottschalk. An update on Streptococcus suis identification. J. Vet. Diagn. Invest., 2 (1990), pp. 249-252

[39] M.B. Marques, D.L. Kasper, M.K. Pangburn, M.R. Wessels. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. Infect. Immun., 60 (1992), pp. 3983-3986

[40] H.K. Guttormsen, L.C. Paoletti, K.G. Mansfield, W. Jachymek, H.J. Jennings, D.L. Kasper. Rational chemical design of the carbohydrate in a glycoconjugate vaccine enhances IgM-to-IgG switching. Proc. Natl. Acad. Sci. USA., 105 (2008), pp. 5903-5908

[41] N. Charland, M. Kobish, B. Martineau-Doizé, M. Jacques, M. Gottschalk. Role of capsular sialic acid in virulence and resistance to phagocytosis of Streptococcus suis capsular type 2. FEMS Immunol. Med. Microbiol., 14 (1996), pp. 195-203

[42] A.L. Lewis, V. Nizet, A. Varki. Discovery and characterization of sialic acid O-acetylation in group B Streptococcus. Proc. Natl. Acad. Sci. USA., 101 (2004), pp. 11123-11128

[43] T.L. Wilson, J. Jeffers, V.J. Rapp-Gabrielson, S. Martin, L.K. Klein, D.E. Lowery, T.E. Fuller. A novel signature-tagged mutagenesis system for Streptococcus suis serotype 2. Vet. Microbiol, 122 (2007), pp. 135-145

[44] J.A. Bozue, M.V. Tullius, J. Wang, B.W. Gibson, R.S. Munson Jr. Haemophilus ducreyi produces a novel sialyltransferase. Identification of the sialyltransferase gene and construction of mutants deficient in the production of the sialic acid-containing glycoform of the lipooligosaccharide. J. Biol. Chem., 274 (1999), pp. 4106-4114

[45] M. Katsumi, T. Saito, Y. Kataoka, T. Itoh, N. Kikuchi, T. Hiramune. Comparative preparation methods of sialylated capsule antigen from Streptococcus suis type 2 with type specific antigenicity. J. Vet. Med. Sci., 58 (1996), pp. 947-952

Tables

Table 1. Bacterial strains and plasmids used in this study.

Strains/plasmids	General characteristics	Source/reference
Escherichia coli		
TOP 10	F-*mrcA* Δ(*mrr-hsd*RMS-*mcr*BC)*ϕ80 lacZΔM5* Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *galU galK rpsL* (StrR) *end*A1 *nup*G	Invitrogen
MC1061	*araD139 Δ(ara-leu)7697 ΔlacX74 galU galK hsdR2*(rK- mK+) *mcrB1 rpsL*	[18]
Streptococcus suis		
P1/7	Wild type, highly virulent serotype 2 strain isolated from a pig with meningitis	[19]
Δ*neuC*	Asialo mutant strain derived from P1/7. In frame deletion of *neuC*	This work
Δ*neuC*/*neuC*	Δ*neuC* complemented with pCompNeuc	This work
Δ*cpsF*	Non-encapsulated mutant strain derived from P1/7. In frame deletion of *cpsF*	[7]
S735	Serotype 2 reference strain isolated from a pig with meningitis	[20]
BD101	Non-encapsulated strain derived from S735.	[21]
Group B *Streptococcus*		
COH-1	Wild type, highly encapsulated type III strain isolated from an infant with bacteraemia	[22]
Δ*neuB*	Asialo mutant strain derived from COH-1. In frame deletion of *neuB*	This work
Δ*cpsE*	Non-encapsulated mutant strain derived from COH-1. In frame deletion of *cpsE*	This work
Plasmids		

pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Invitrogen
pSET4s	Thermosensitive vector for allelic replacement in *S. suis* and GBS. Replication functions of pG + host3, MCS oriR pUC19 *lacZ* Sp^R	[23]
p4ΔneuC	pSET4s carrying the construct for *neuC* allelic replacement	This work
p4ΔcpsE	pSET4s carrying the construct for *cpsE* allelic replacement	This work
p4ΔneuB	pSET4s carrying the construct for *neuB* allelic replacement	This work
pMX1	Replication functions of pSSU1, MCS pUC19 *lacZ* Sp^R, *malX* promoter of *S. suis*, derivative of pSET2	[24]
pCompNeuC	pMX1 carrying the entire *neuC* gene under the control of pMalX	This work

Table 2. Oligonucleotide primers used in this study. [a]

Primer name	Sequence (5'-3')
For construction of in-frame deletion mutants	
neuC-ID1	TGCCCGTTTATAAGATTCCATC
neuC-ID2	TGAGTTGCTCTGTCAAGGTC
neuC-ID3	TGATTGAAGTGCCCTCATTAC
neuC-ID4	TAAACCTTTTGATCCTGACCG
neuC-ID5	TGAAAAGCACTTTACTCTGGAC
neuC-ID6	CCTTGTAAAGCAGAATCAGGTTGATGCATGGCTGTCACTAC
neuC-ID7	GTAGTGACAGCCATGCATCAACCTGATTCTGCTTTACAAGG
neuC-ID8	ATGTTCCACAATGGCACCC
GBScpsE-ID1	AGAATACTTCAATGCGATCCG
GBScpsE-ID2	TCAAGATAGCCACGACTCC
GBScpsE-ID3	AAGGCGATATGAGTTTAGCAG
GBScpsE-ID4	CGCCATGTGTGATAACAATCTC
GBScpsE-ID5	TGGAACTATTAAAGGCTTGACG
GBScpsE-ID6	CCTGTCCCGAGTAAAACTACTACAACTGTTTGAATCATCGC
GBScpsE-ID7	GCGATGATTCAAACAGTTGTAGTAGTTTTACTCGGGACAGG
GBScpsE-ID8	TCCCCACTGTGACAAAAATC
GBSneuB-ID1	TCTAGGGTTTTTGGAGCTTTTG
GBSneuB-ID2	ACAGCATCAACACCACAAG
GBSneuB-ID3	TGGATCAGAAGTACCTATCGC
GBSneuB-ID4	GGTTGTTCTCCCATCTGAATC
GBSneuB-ID5	GCTCAAATAATGGGAGGAGAC
GBSneuB-ID6	GTCCCAAGATGTCATACCAGTGATTGCAACCAATCTCTGC
GBSneuB-ID7	GCAGAGATTGGTTGCAATCACTGGTATGACATCTTGGGAC
GBSneuB-ID8	TTCGTAACGATCCCCTAAAATG
For construction of complemented mutants [b]	
neuC-CF	TGAG**CTGCAG**CAAAATATTTGCCATAGTGC
neuC-CR	CAT**CTGCAG**AGGTACCCGCTCCTAGAAAGG

[a] Oligonucleotide primers were from Invitrogen.

[b] PstI restriction sites indicated in bold.

Figures

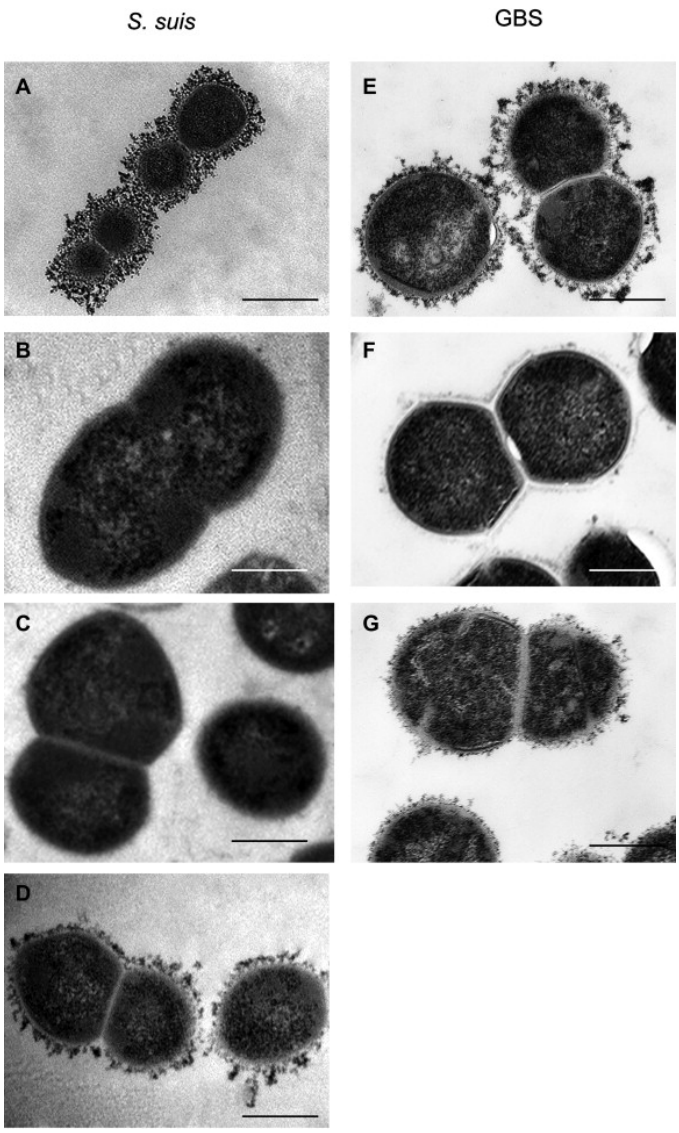


Figure 1. Transmission electron micrographs showing capsular polysaccharide (CPS) expression by different strains of *S. suis* and Group B Streptococcus (GBS).

(A) S. suis wild type strain P1/7 is surrounded by a thick capsule whereas the Δ*cpsF* and the Δ*neuC* mutant strains are non-encapsulated (B–C). The Δ*neuC*/*neuC* strain shows intermediate CPS expression (D). GBS wild type strain COH-1 is surrounded by a thick capsule (E) whereas the Δ*cpsE* strain is non-encapsulated (F). The Δ*neuB* strain presented some residual CPS (G). Bars = 0.5 μm.

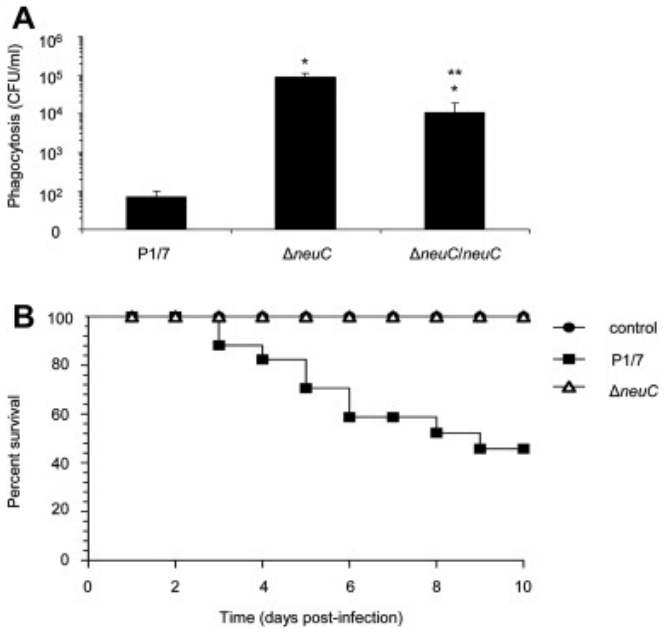


Figure 2. Effect of the deletion of *neuC* on the resistance of *S. suis* to phagocytosis by macrophages and on virulence.

(A) Phagocytosis studies with J774 macrophages and different strains of *S. suis*. Bacteria were incubated with macrophages for 90 min. Numbers of internalized bacteria were determined by quantitative plating after 1 h of antibiotic treatment, and the results are expressed as CFU recovered bacteria per ml (means ± SEM obtained from four independent experiments). *$P < 0.05$, indicates statistically significant differences between the wild type strain and the Δ*neuC* mutant and Δ*neuC*/*neuC* strains. **$P < 0.05$, indicates statistically significant differences between the Δ*neuC* mutant strain and the Δ*neuC*/*neuC* strain. (B) Survival of mice infected with the wild type P1/7 strain and its Δ*neuC* mutant. All mice in the Δ*neuC* mutant group (n = 15) survived, whereas 53% of the wild type group (n = 15) died from septicemia or meningitis. Significant differences in survival were noted (LogRank test, $P < 0.05$).

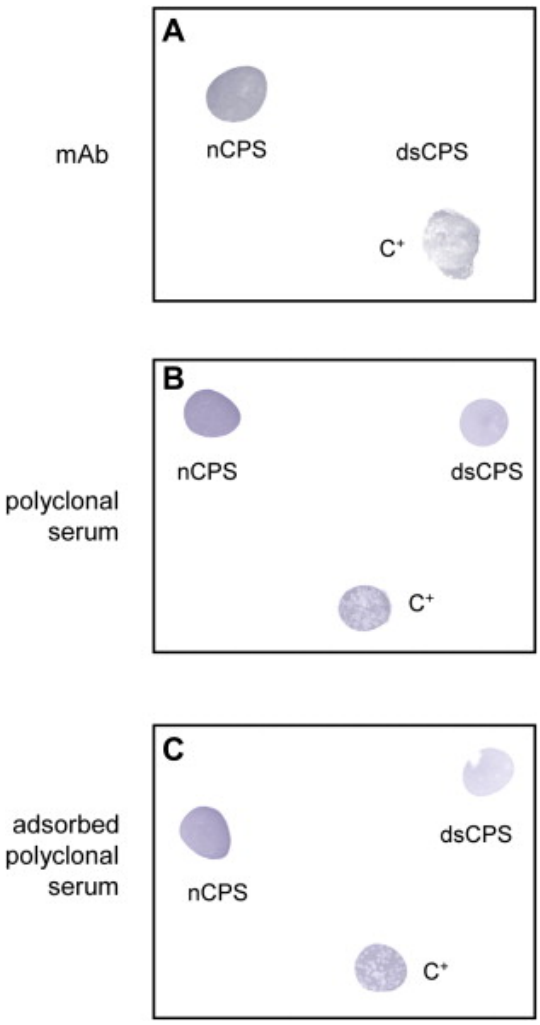


Figure 3. Role of capsular polysaccharide (CPS) sialylation as the main capsular epitope of *S. suis* serotype 2.

Whole bacteria (C^+) and native (nCPS) or desialylated purified CPS (dsCPS) were incubated with a monoclonal antibody directed against the sialic acid moiety of the CPS, total polyclonal anti-*S. suis* serotype 2 serum or a monospecific adsorbed polyclonal antibody anti-*S. suis* serotype 2 CPS. (A) The monoclonal antibody recognized the native CPS and whole-cell bacteria. (B) The polyclonal anti-*S. suis* serotype 2 serum recognized whole-cell bacteria and both the native and desialylated CPS. (C) The adsorbed monospecific polyclonal anti-*S. suis* serotype 2 CPS serum also recognized whole-cell bacteria and both the native and desialylated CPS.

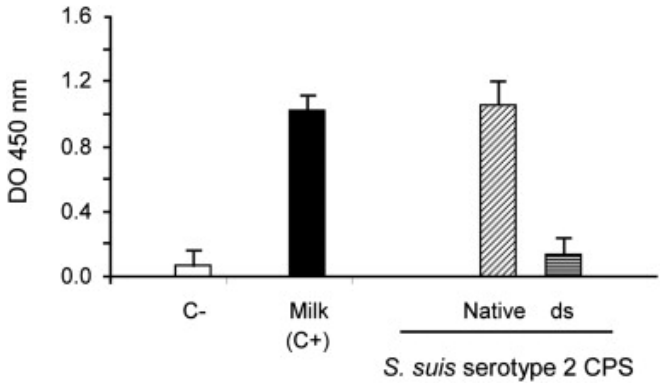


Figure 4. Recognition of *S. suis* sialic acid by *Sambucus nigra* lectin (SNA-I).

SNA-I Enzyme Linked Lectin Assay showed that native capsular polysaccharide (CPS), but not desialylated CPS (dsCPS), was clearly recognized by lectin. Dilution buffer was used as negative control (C-) and skimmed milk was used as positive control (C+).

Supporting information

Supplementary material is available with the article through the journal Web site at https://www.sciencedirect.com/science/article/pii/S1286457912000901?via%3Dihub#appsec1

Supplementary Table S1. Homology between *Streptococcus suis* and GBS proteins encoded by *neu* locus.

ANNEXES - ARTICLE XV

Group B *Streptococcus* and *Streptococcus suis* Capsular Polysaccharides Induce Chemokine Production By Dendritic Cells Via TLR2- and MyD88-Dependent and -Independent Pathways

---

Cynthia Calzas [1], **Guillaume Goyette-Desjardins** [1], Paul Lemire [1], Fleur Gagnon [2], Claude Lachance [1], Marie-Rose Van Calsteren [2], and Mariela Segura [1]

[1] Laboratory of Immunology, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, Quebec, Canada

[2] Food Research and Development Centre, Agriculture and Agri-Food Canada, Saint-Hyacinthe, Quebec, Canada

Publié dans *Infection and Immunity*. **2013**. 81(9):3106-3118.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé partiellement aux expériences de purification et désialylation (20%).

Abstract

Streptococcus agalactiae (also known as Group B *Streptococcus* or GBS) and *Streptococcus suis* are encapsulated streptococci causing severe septicemia and meningitis. Bacterial capsular polysaccharides (CPSs) are poorly immunogenic, but anti-CPS antibodies are essential to the host defense against encapsulated bacteria. The mechanisms underlying anti-CPS antibody responses are not fully elucidated, but the biochemistry of CPSs, particularly the presence of sialic acid, may have an immunosuppressive effect. We investigated the ability of highly purified *S. suis* and GBS native (sialylated) CPSs to activate dendritic cells (DCs), which are crucial actors in the initiation of humoral immunity. The influence of CPS biochemistry was studied using CPSs extracted from different serotypes within these two streptococci, and desialylated CPSs. No IL-1β, IL-6, IL-12p70, TNF-α or IL-10 production was observed by *S. suis* or GBS CPS-stimulated DCs. Moreover, these CPSs exerted immunosuppressive effects on DC activation, as a diminution of IFN-γ-induced B cell-activating factor of the tumor necrosis factor family (BAFF) expression was observed in CPS-pretreated cells. However, *S. suis* and GBS CPSs induced significant production of CCL3, via partially TLR2 and MyD88-dependent pathways, and CCL2, via TLR-independent mechanisms. No major influence of CPS biochemistry was observed on the capacity to induce chemokine production by DCs, indicating that DCs respond to these CPSs in a pattern way rather than in a structure-dedicated manner.

Introduction

Streptococcus agalactiae (also known as Group B *Streptococcus* or GBS) is a major cause of life-threatening invasive bacterial infections in pregnant women and neonates, as well as the elderly and immunocompromised individuals (1, 2). Clinical manifestations are mainly pneumonia, septicemia, and meningitis. Among ten GBS serotypes identified, type III is one of the major serotypes associated with invasive neonatal infection and is the most common type in GBS meningitis (2). In addition, GBS type V is emerging as a leading cause of invasive disease in adults (3). *Streptococcus suis* is an important swine and an emerging zoonotic pathogen in humans which is able to induce septicemia with sudden death, meningitis, endocarditis, pneumonia, and arthritis (4, 5). Of the 35 serotypes, type 2 is the most virulent and frequently isolated from both swine and humans (6) and type 14 is also emerging as a zoonotic threat (7). For both pathogens, the capsular polysaccharide (CPS), which defines the serotype, is considered as the major virulence factor (8, 9). The structures of types III and V GBS CPSs are formed by different arrangements of the monosaccharides glucose, galactose, and *N*-acetylglucosamine into unique repeating units that contain a side chain terminated by sialic acid (*N*-acetylneuraminic acid; Neu5Ac). The structures of types 2 and 14 *S. suis* CPSs are composed of the monosaccharides glucose, galactose, *N*-acetylglucosamine, and rhamnose (for type 2 only) arranged into a unique repeating unit that also contains a side chain terminated by sialic acid. In fact, these streptococci are the sole Gram-positive bacteria possessing sialic acid in their capsules (8, 10, 11). However, despite similarities in the composition of the CPSs of these two bacterial species, each CPS is composed of a unique arrangement of these sugars conferring a distinct antigenicity. Moreover, sialic acid forms an α-2,6 linkage with the adjacent galactose in *S. suis versus* an α-2,3 linkage in GBS. Interestingly, their interplay with components of the immune system, including antigen-presenting cells (APCs) seems to radically differ. Experiments using non-encapsulated mutants have shown that *S. suis* type 2 CPS has a strong anti-phagocytic effect contrary to GBS type III CPS and severely interferes with the release of most of the cytokines produced by *S. suis*-infected APCs. In the case of GBS type III, cytokine production is only partially modified or unaltered by the presence of its CPS (12-15).

Sialic acid of bacterial polysaccharides has been suggested to be involved in immune evasion via several mechanisms. For example, sialic acid of GBS type III CPS interferes with the immune response by molecular mimicry (8) and inhibition of complement activation (16). Some receptors expressed on the surface of leukocytes have a distinct preference for specific types of linkage of sialic acid to subterminal sugars. As these binding preferences are likely related to their biological functions, differences in sialic acid linkage in *S. suis versus* GBS might differentially modulate host immune responses (17). However, knowledge on the specific contribution of sialic acid to the interactions of these two pathogens with the immune system is restricted by the fact that deletion of genes involved in sialic acid synthesis results in considerable or complete loss of CPS expression at the bacterial surface (18, 19).

Dendritic cells (DCs), the most powerful APCs, express a wide variety of pattern-recognition receptors (PRRs) that enable them to detect the presence of several pathogens through the recognition of pathogen-associated molecular patterns. Among these PRRs, toll-like receptors (TLRs) are important for the initiation of the immune response as well as the shaping of adaptive immunity (20). The interactions between DCs and pathogens can strongly influence the magnitude and phenotype of the ensuing cellular and humoral adaptive immune response, notably via the release of cytokines (21). Purified bacterial CPSs are classically reported to be T cell-independent (TI) antigens which are consequently weak stimulators of the host immune response (22, 23). However, several *in vitro* studies have demonstrated the ability of bacterial CPSs to interact with APCs, resulting in the production of cytokines and chemokines (24-28). The adaptor molecule myeloid differentiation factor 88 (MyD88), which is involved in intracellular events downstream of TLR signaling, and TLR2 have been suspected to be involved in the interactions of bacterial CPSs with DCs and/or macrophages (25, 27). Nevertheless, the potential role of pure carbohydrates as ligands for TLRs, and more globally for PRRs, remains largely uninvestigated.

Recent reports have shown that DCs play an important role in TI responses, and more precisely in the development of the humoral response, via the release of B cell-activating factor of the tumor necrosis factor family (BAFF). BAFF is able to enhance B cell proliferation, immunoglobulin (Ig) class switching and Ig secretion (29, 30). These critical

signals are particularly interesting in the context of infection to encapsulated bacteria, where antibodies (Abs) against the CPS are proven essential to the host defense (23). As such, we hypothesized that intra- and inter-species structural differences in CPS might differently modulate DC release of cytokines and chemokines. The goal of this study was thus to evaluate and compare the effect of highly purified CPS preparations from *S. suis* types 2 and 14 as well as GBS types III and V on DC activation, and more specifically on the capacity to induce cytokines essential for the development of effective humoral immune response. The influence of sialic acid was analyzed using chemically desialylated CPS preparations.

Material and Methods

Native CPS purification.

The reference strains of *S. suis* serotype 2 S735 (ATCC 43765), isolated from a pig with meningitis (31), and of *S. suis* serotype 14 DAN13730, isolated from a case of human meningitis (32), were grown in 150 ml of Todd-Hewitt broth (THB) (Oxoid, Thermo Fisher Scientific) at 37°C for 16 h, diluted to 6 l in fresh THB, and grown to an optical density at 540 nm ($OD_{540\ nm}$) of 0.8. The cells were pelleted by centrifugation at 10,000 × *g* for 40 min, suspended by repeated pipetting in 33 mM phosphate-buffered saline (PBS) pH 8.0, and chilled. The CPSs were then purified as previously described (10, 11).

GBS serotype III strain COH-1, isolated from an infant with bacteremia (33), and GBS serotype V strain CJB111 (ATCC BAA-23), isolated from a neonate with septicemia, were used in this study. GBS CPSs were prepared as previously reported (34) with some modifications. Briefly, bacteria were grown in 200 ml THB at 37°C for 16 h, diluted to 8 l in fresh THB, and grown to an $OD_{540\ nm}$ of 0.8. The cells were pelleted by centrifugation at 10,000 × *g* for 40 min, washed in PBS pH 7.3 and treated with 1 N NaOH at 37°C overnight. After neutralization and dialysis, proteins were digested by 1 mg/ml pronase treatment (Sigma-Aldrich) at 37°C overnight, followed by dialysis. The CPSs were then subjected to re-*N*-acetylation with 0.8 M acetic anhydride (Sigma) in 5 N NaOH and finally purified by gel filtration on Sephacryl S-300 (GE Healthcare) using 50 mM NH_4HCO_3 as the eluent.

CPS desialylation.

Highly purified native CPSs were desialylated by mild acid hydrolysis as described previously (10). Briefly, the CPS (8 mg) was heated in 1 ml of 70 mM HCl at 60°C for 250 min, neutralized with 2 M NH_4OH, dialyzed against deionized water for 48 h at 4°C with a Spectra/Por membrane MWCO 3500 Da (Spectrum Laboratories), and freeze dried.

CPS quality controls.

Each purified CPS was subjected to rigorous quality control tests as previously described (10). Nucleic acids were quantified using an ND 1000 spectrometer (Nanodrop). The absorbance was measured at 230 and 260 nm. Calculations were done with the Nanodrop software. According to the manufacturer, results are reproducible between 2 and 100 ng/µl. Proteins were quantified by the modified Lowry protein assay kit from Pierce on 1 mg/ml CPS samples using a standard curve prepared with diluted albumin standards from 1 to 1000 µg/ml. The calculated limit of detection ($P \leq 0.05$) was 0.7-1.3 µg/ml. Each CPS was analyzed by nuclear magnetic resonance (NMR) as described below. The monosaccharide composition of polysaccharides was confirmed by methanolysis followed by acetylation and analysis by gas chromatography (GC) either with flame ionization detection or coupled to mass spectrometry as previously described (10). Weight-average molecular mass (M_w) of each CPS was determined by size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) as described below. Presence (native CPS) or absence (desialylated CPS) of sialic acid was verified by NMR and by an enzyme-linked lectin assay (ELLA) as described below.

Nuclear magnetic resonance.

S. suis native CPSs were exchanged in phosphate buffer (p^{2}H 8.0) in ^2H_2O (99.9 atom% ^{2}H), freeze dried, and dissolved in ^2H_2O (99.96 atom% ^{2}H) to a final phosphate concentration of 33 mM. The other CPSs were exchanged in ^2H_2O (99.9 atom% ^{2}H), freeze dried, and dissolved in ^2H_2O (99.96 atom% ^{2}H). NMR spectra were acquired on CPS samples at concentrations of circa 1%–2%. Conventional ^{1}H spectra were acquired at 14 T on Bruker Avance spectrometers equipped with either a 5 mm TCI CryoProbe at 50°C or a 5 mm PABBO BB inverse gradient probe at 75°C or at 11.75 T on a Bruker Avance 500

spectrometer equipped with a 5 mm triple-resonance TBI probe at 60–80°C using standard Bruker pulse sequences.

Weight-average molecular mass characterization of native and desialylated CPSs.

M_w of CPSs were characterized by SEC-MALS. The chromatographic separation was performed with two 8 mm × 300 mm Shodex OHpak gel filtration columns connected in series (SB 806 and SB 804), preceded by an SB 807G guard column (Showa Denko). Elution was done with a Waters 510 pump (Waters) using a 0.1 M $NaNO_3$ mobile phase filtered through a 0.02 µm membrane (Whatman), at a flow rate of 0.5 ml/min. Samples were dissolved in the SEC eluent at a concentration of 0.7-1.0 mg/ml for the native CPS and 2.0-3.5 mg/ml for the desialylated CPS, and injected with a 100 or 200 µl sample loop. Molecular masses were determined with a Dawn EOS MALS detector (Wyatt). A differential refractometer model RI 410 (Waters) was used as a concentration detector. A refractive index increment (*dn*/*dc*) of 0.137 ml/g was calculated for 690 nm using data for xanthan at 436 and 546 nm (35), and the second virial coefficient (A_2) was taken as zero. Calculations were performed with the ASTRA software version 6.0.0.108 (Wyatt).

Enzyme-linked lectin assay (ELLA).

In order to verify the presence or absence of sialic acid in the purified native and desialylated CPSs, an ELLA test was carried out based on a previously described technique (36), which was adapted to the CPSs. Briefly, 200 ng of sample (native or desialylated CPS) was added to wells of an enzyme-linked immunosorbent assay (ELISA) plate (Nunc-Immuno Polysorp). After overnight coating at 4°C, the wells were washed and blocked by the addition of Carbo-Free solution 1X (Vector Labs). After washings, the wells were incubated 1 h with biotinylated *Sambucus nigra* agglutinin (SNA-I) (Vector Labs), which specifically recognizes sialic acid as Neu5Acα-2,6-Gal*p*/Gal*p*NAc (37), or biotinylated *Maackia amurensis* leukoagglutinin (MAL-I) (Vector Labs) recognizing sialic acid as Neu5Acα-2,3-Galβ-1,4-GlcNAc (38). Then, horseradish peroxidase (HRP)-labeled Avidin D (Vector Labs) and 3,3′,5,5′-tetramethylbenzidine were added. In some experiments, HRP-conjugated *Limax flavus* agglutinin (LFA) (Vector Labs), which recognizes Neu5Ac (39), was used. The enzyme

reaction was stopped with the addition of 0.5 M H_2SO_4 and the absorbance was read at 450 nm with an ELISA plate reader.

Dot-ELISA.

Ten µl of purified native or desialylated CPS (each at 1 mg/ml) or 10 µl of heat-killed whole bacteria were blotted on a PVDF Western blotting membrane (Roche). Heat-killed bacteria were obtained after incubating bacteria at 60°C for 45 min and were adjusted to 10^9 CFU/ml. The membrane was blocked for 1 h with a solution of Tris-buffered saline (TBS) containing 2% casein, followed by a 2 h incubation with either the mouse monoclonal Ab (mAb) Z3 which specifically recognizes the sialic acid moiety of *S. suis* type 2 CPS (40), mono-specific polyclonal rabbit sera against *S. suis* type 2 (41) or *S. suis* type 14 (32), or commercial rabbit sera against GBS type III or GBS type V CPS (Denka Seiken). The membrane was washed and the appropriate anti-rabbit or anti-mouse HRP-conjugated Ab (Jackson) was added for 1 h. The membrane was washed 3 times with TBS and revealed with a 4-chloro-1-naphthol solution (Sigma).

Mouse strains and generation of bone marrow-derived dendritic cells.

Six- to eight-week-old mice originating from Jackson Laboratory including wild type (WT) C57BL/6, MyD88$^{-/-}$ (B6.129P2-*Myd88*^{tm1Defr}/J), and TLR2$^{-/-}$ (B6.129-*Tlr2*^{tmlKir}/J) mice were used. All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals by the Animal Welfare Committee of Université de Montréal. Bone marrow-derived DCs were produced according to a technique previously described (13, 42) and cultured in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 U/ml penicillin-streptomycin and 20 µg/ml gentamycin. All reagents were from Gibco (Invitrogen). Cell purity was routinely ≥ 86-90% CD11c^{+high} and F4/80$^{-/dim}$ cells as determined by FACS analysis, values that were in agreement with those reported in other studies (43-45).

In vitro DC stimulation assay.

DCs were resuspended at 10^6 cells/ml in complete medium and stimulated with native or desialylated CPS (5, 50, 100, 200 µg/ml). At 6 and 24 h, supernatants were collected for cytokine quantification by ELISA and cells were harvested for analysis of BAFF expression by reverse transcriptase-quantitative PCR (RT-qPCR). Cells stimulated with 1 µg/ml ultra-purified *Escherichia coli* O55:B5 lipopolysaccharide (LPS) (Apotech Corporation) or 10 ng/ml recombinant mouse IFN-γ (R&D Systems) served as positive control for cytokine production and BAFF expression, respectively. Non-stimulated cells served as negative control. In some experiments, DCs were pre-stimulated with CPS (at 200 µg/ml) for 6 h prior to incubation with 10 ng/ml IFN-γ for 24 h. Cells were then harvested for BAFF expression analysis. DCs pre-incubated in complete medium before addition of IFN-γ served as control. All solutions and CPSs were tested for the absence of endotoxin using a *Limulus* amebocyte lysate gel-clot test (Pyrotell, STV) with a sensitivity limit of 0.03 EU/ml. Otherwise, absence of endotoxin contamination during cell stimulation was controlled by parallel assays with polymyxin B sulfate (Sigma) at 20 µg/ml.

Cytokine quantification by ELISA.

Levels of IL-1β, IL-6, IL-10, IL-12p70, TNF-α, CCL2 (MCP-1) and CCL3 (MIP-1α) in cell culture supernatants were measured by sandwich ELISA using pair-matched Abs from R&D Systems according to the manufacturer's recommendations. Two-fold dilutions of recombinant mouse cytokines were used to generate standard curves. Sample dilutions giving OD readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine.

Analysis of BAFF gene expression by real time RT-qPCR.

Total RNA was isolated from 10^6 DCs with the Trizol reagent (Invitrogen). After elimination of genomic DNA, 800 ng of total RNA was reverse-transcribed with the QuantiTect reverse transcription kit (Qiagen). The cDNA was amplified using the SsoFast™ EvaGreen® Supermix kit (Bio-Rad). The PCR amplification program for all cDNA consisted of an enzyme activation step of 3 min at 98°C, followed by 40 cycles of a denaturing step of 2 s at 98°C and an annealing/extension step of 5 s at 56°C. ATP synthase subunit beta (ATP5B)

and cytochrome c1 (CYC1) were used as normalizing genes to compensate for potential differences in cDNA amounts. The primers used for amplification of the different target cDNA are listed in Table S1 and were all tested to achieve an amplification efficiency between 98.5% and 100%. The primer sequences were all designed from the NCBI GenBank mRNA sequence using the web-based software primerquest from Integrated DNA technologies. The Bio-Rad CFX-96 sequence detector was used for amplification of cDNA, and quantitation of differences between the different groups was calculated using the $2^{-\Delta\Delta CT}$ method (46). Non-stimulated DCs were used as the calibrator reference in the analysis.

Statistical analysis.

All data are expressed as mean ± SEM. Data were analyzed for significance using ANOVA test. Significance is denoted in the figures (*, $P < 0.05$; **, $P < 0.001$). All experiments were repeated at least three times.

Results

S. suis and GBS CPS purification, desialylation and quality control tests.

On average, from a 6 l of *S. suis* type 2 or *S. suis* type 14 culture, 150 mg of crude capsule was obtained to afford around 30 mg of purified CPS after gel filtration (average yield of 5 mg CPS/l of culture). From 8 l of GBS type III or type V culture, 360 mg or 600 mg initial crude capsule was obtained respectively, to afford around 30 mg of purified CPS after gel filtration (average yield of 3.75 mg CPS/l of culture). GC and NMR analysis of purified CPSs gave sugar composition and structure in accord with previous findings (8, 10, 11) (Figs. S1 and S2; Table 1). Each repeating unit is composed of the same four sugars (with an additional rhamnose in *S. suis* type 2 CPS), where sialic acid is located at the terminal side chain. The ratio of the four common sugars is similar in *S. suis* types 2 and 14 CPSs, whereas it is different between GBS types III and V CPSs. In comparison with *S. suis* CPSs, the repeating unit of GBS types III and V CPSs has one less galactose, and GBS type V CPS has two additional glucose residues. Indeed, the sugar ratio of glucose:galactose:*N*-acetylglucosamine:*N*-acetylneuraminic acid:rhamnose for each repeating unit of *S. suis* type 2, *S. suis* type 14, GBS type III and GBS type V CPSs is 1:3:1:1:1, 1:3:1:1:0, 1:2:1:1:0 and

3:2:1:1:0, respectively (Figs. S1 and S2; Table 1). One of the problems encountered when purifying GBS CPSs is contamination with group B antigen, as the CPS is covalently linked to the cell wall in this bacterial species (47). Absence of rhamnose (a sugar present in group B antigen but not in GBS CPSs) in purified GBS CPS preparations confirmed the absence of contamination with this cell wall antigen. No protein was found above the limit of detection, indicating that there was less than 1.3% weight/weight protein (w/w protein) in all purified CPSs, and DNA and RNA contamination was less than 1% w/w (Table 1). SEC-MALS showed that native *S. suis* types 2 and 14 CPSs had a similar M_w, which was 480 and 500 kDa, respectively. Native GBS types III and V CPSs had comparable M_w, which was almost 4-fold lower than that of native *S. suis* CPSs. Desialylation induced a higher M_w decrease in *S. suis* CPSs than in GBS CPSs, with the highest diminution for desialylated *S. suis* type 2 CPS (96% of the initial M_w) (Table 1). This could be explained by the presence of rhamnose in the backbone of *S. suis* type 2 CPS, which forms a linkage more susceptible to acid hydrolysis with the adjacent sugar.

S. suis and GBS CPS recognition by specific sera and sialic acid-binding lectins.

Dot-ELISA experiments on native CPSs showed that recognition of the CPS epitope of each CPS preparation was conserved after purification (Fig. 1). Whereas the native *S. suis* type 2 CPS was well recognized by mAb Z3, which is specific for the sialic acid part of the capsule, the negative reaction of the desialylated *S. suis* type 2 CPS attested the absence of sialic acid in the latter (Fig. 1A, left). Desialylation of *S. suis* type 2 CPS resulted in reduced recognition by specific polyclonal Abs (Fig. 1A, right), whereas recognition of desialylated preparations of *S. suis* type 14 CPS was unaltered (Fig. 1B). The capacity of specific polyclonal Abs to react with the desialylated GBS type III CPS was almost completely lost compared to the native CPS (Fig. 1C). In contrast, native and desialylated preparations of GBS type V CPS were similarly recognized by specific polyclonal Abs (Fig. 1D). These data suggest that there are intra- and inter-species variations in the immunogenic properties exerted by the sialic acid moiety (48-50).

To further confirm the presence or absence of sialic acid in native and desialylated CPSs, an ELLA test was performed. Recognition of native *S. suis* and GBS CPSs by LFA,

which is specific for Neu5Ac, confirmed the integrity of sialic acid in these preparations, whereas the absence of reaction with desialylated preparations demonstrated the absence of this sugar after desialylation by mild acid hydrolysis (Fig. 2A). Recognition of native *S. suis* CPSs by SNA-I and native GBS CPSs by MAL-I validated that sialic acid forms an α-2,6 and an α-2,3 link with the adjacent galactose, respectively (Fig. 2B and C). In the case of desialylated *S. suis* type 14 CPS, a positive reaction was observed with both SNA-I and MAL-I. Similarly, a positive reaction was observed for desialylated GBS type III CPS with MAL-I (Fig. 2B and C). As negative reactions with LFA and NMR analysis (Figs. S1, S2 and 2A) clearly demonstrated the absence of sialic acid in these desialylated preparations, the positive reaction with SNA-I can be explained by the non-specific binding of the lectin with D-galactose (37). Recognition of Galβ-1,4-GlcNAc epitope of desialylated *S. suis* type 14 and GBS type III CPSs by MAL-I can explain the unspecific reaction of this lectin with these two preparations (51).

S. suis and GBS CPSs induce the release of chemokines by DCs.

The levels of several cytokines and chemokines in the supernatants of DCs incubated with *S. suis* types 2 or 14 or GBS types III or V CPS (each at 200 µg/ml) were measured at 24 h after stimulation. No significant difference in pro- or anti-inflammatory cytokine production was observed between DCs incubated with the different CPSs and those incubated with medium alone (Fig. 3). Similar results were obtained at 6 h after stimulation (data not shown). Presence of sialic acid did not influence the release of these cytokines, as no significant difference was observed between native and desialylated CPSs (Fig. 3).

In contrast, and interestingly, *S. suis* and GBS CPSs induced significant release of the chemokines CCL2 and CCL3 at 24 h (Fig. 4). CCL3 production was similarly induced by all CPS preparations (Fig. 4A). On the other hand, CCL2 production was significantly higher when DCs were activated with *S. suis* CPSs ($P < 0.001$; Fig. 4B). In the case of *S. suis*, a higher CCL2 production was observed by DCs stimulated with *S. suis* type 14 CPS in comparison to *S. suis* type 2 CPS ($P < 0.001$). Sialic acid plays a partial inhibitory role in the production of CCL2 for *S. suis* type 2 CPS only ($P < 0.001$). To explore more precisely the characteristics of CCL2 and CCL3 production, dose- and time-response analyses were

performed. CCL2 release induced by *S. suis* type 2 CPS was shown to be directly proportional to the CPS concentration and the time of incubation, with maximum release obtained at 24 h when using a concentration of 200 µg/ml CPS (Fig. 5). Similar results were obtained with *S. suis* type 14, GBS types III and V CPSs, for both CCL2 and CCL3 production (data not shown).

Involvement of PRRs in chemokine release by DCs stimulated with S. suis and GBS CPSs.

Previous *in vitro* studies have demonstrated that well-encapsulated *S. suis* type 2 induces TLR2 mRNA expression by human monocytes (52) and porcine DCs (15) and stimulates cytokine and chemokine production by murine macrophages and DCs in a TLR2- and MyD88-dependent manner (52, 53). Moreover, the release of cytokines by *S. suis*-stimulated human monocytes was significantly reduced by Ab-mediated blocking of TLR2 but not TLR4 (52). In the case of GBS type III, killed bacterium induces TLR2 mRNA expression and TLR2-dependent expression of certain cytokines and chemokines by murine macrophages (54). Killed GBS type III stimulates TNF-α release in a TLR2-independent but MyD88-dependent way by those cells (55). As earlier studies have shown that PPRs like TLRs are involved in cytokine production by APCs stimulated with purified CPSs from several different bacteria (25, 27, 56), we aimed to evaluate the implication of these receptors in the production of chemokines by DCs activated with our CPSs. Therefore, CCL2 and CCL3 release was compared between WT and TLR2$^{-/-}$or MyD88$^{-/-}$ DCs incubated with *S. suis* or GBS CPSs (at 200 µg/ml) for 24 h. As shown in Figs. 6A and 7A, no significant difference in CCL2 production was observed between WT and TLR2$^{-/-}$ or between WT and MyD88$^{-/-}$ DCs for either *S. suis* or GBS CPSs. On the other hand, TLR2$^{-/-}$ DCs activated with either GBS type III or type V CPS showed a partial reduction of CCL3 production than WT DC counterparts ($P < 0.05$). In the case of *S. suis*, a significant effect of TLR2 on CCL3 production was only observed for native *S. suis* type 2 CPS, indicating the involvement of other receptors (Fig. 6B). Indeed, a significant impairment of CCL3 production by MyD88$^{-/-}$ DCs incubated with all four *S. suis* or GBS CPSs was observed, with a decrease varying between 40 and 50% ($P < 0.001$) (Fig. 7B). In general, the presence of sialic acid did not seem to significantly modulate the interactions between either GBS or *S. suis* CPSs with the TLR/MyD88 pathway (Figs. 6 and 7).

Modulation of BAFF gene expression by DCs incubated with S. suis and GBS CPSs.

In contrast to the numerous studies that have focused on the effect of BAFF on B cell physiology, there is a relative paucity of evidence concerning BAFF production by DCs. A recent study has shown that the prototype TI antigen NP-Ficoll is able to induce the release of BAFF by murine DCs, which is essential for the development of Ab response (57). To analyze whether *S. suis* and GBS CPSs modulate BAFF mRNA expression levels, DCs were stimulated with native CPSs at 6 and 24 h. IFN-γ (10 ng/ml), known to stimulate BAFF synthesis by monocytes, macrophages and DCs (29, 58, 59), was used as positive control. As shown in Fig. 8, no significant difference in BAFF expression levels was observed between CPS-activated DCs and control non-stimulated cells, either at 6 or 24 h. Only a slight up-regulation (less than 2-fold increase) of BAFF mRNA expression was observed at 6 h for all native CPS preparations. As sialic acid has been shown to inhibit B cell activation (60), we wanted to know if this sugar could be involved in the inhibition of BAFF expression as well. Sialic acid did not significantly modulated BAFF expression by DCs (Fig. 8).

To further evaluate the capacity of GBS and *S. suis* CPSs to inhibit BAFF expression by DCs, we evaluated the impact of DC pre-incubation with CPS on IFN-γ-induced BAFF. To this aim, DCs were pre-cultured with the different CPSs for 6 h prior to stimulation with IFN-γ for 24 h. A diminution of BAFF mRNA expression ranging from 20 to 40% was observed when DCs were pre-incubated with either *S. suis* or GBS CPSs in comparison with non-pretreated cells, confirming the inhibitory effect of these bacterial CPSs on BAFF expression (Fig. 9).

Discussion

CPS is a crucial component for both *S. suis* and GBS. In addition to forming the basis for serotype designation and being the major virulence factor, it owns, as an immunogen, a high protective potential in the fight against infections by these two streptococci. Indeed, as shown with other encapsulated bacteria like *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*, the specific anti-CPS humoral response could play a decisive

role in host survival to *S. suis* or GBS infections. Previous studies have demonstrated that mouse or pig anti-*S. suis* type 2 CPS Abs had a protective role in homologous opsonophagocytosis *in vitro* assays (40, 61, 62), or during *in vivo* challenge (40, 63). Finally, Abs raised against several GBS CPS serotypes, including serotypes Ia, Ib, II, III and V, are protective against neonatal infections in both animals and humans and exhibit opsonophagocytosis ability *in vitro* (64).

However, *S. suis* and GBS CPSs are poorly immunogenic molecules because they cannot recruit T cell help for B cell functions. Mothers of neonates developing GBS type III disease have low concentrations of anti-CPS Abs in sera at delivery (65), and very few anti-CPS Abs can be detected in pigs infected with *S. suis* type 2 (61, 63). To counter the low immunogenicity of CPSs, researchers have developed conjugate vaccine composed of CPS linked to a carrier protein, as it is the case for GBS. However, efficacy of under-trial GBS conjugate vaccines depends on the CPS serotype included in the preparation. Indeed, CPSs of types Ia, Ib, II and III induce strong protective IgG responses, whereas GBS type V CPS promotes higher concentrations of specific IgM relative to IgG (64). This suggests that composition and/or structure of CPS could influence its immunogenicity. Certain structural features of CPSs, such as variations in repeating unit composition or glycosidic linkage positions, are susceptible to produce different immune responses (66-68).

Despite these observations, very few studies have been dedicated to the characterization of CPS activity on APCs and to the corresponding signaling mechanisms. Some bacterial CPS motifs, including sialic acid, are very similar to molecules expressed by human cells or tissues, which could result in immune evasion through molecular mimicry (68). Therefore, chemical alteration of sialic acid of *N. meningitidis* type B CPS by de-*N*-acetylation or deletion of this sugar in GBS type V CPS improved immunogenicity of these two CPSs (49, 69). However, consequences of capsular sialic acid manipulation on the CPS capacity to activate the immune system have been only evaluated in the context of Ab production. The mechanisms underlying this effect, such as the modulation of the function of APCs, are poorly known.

In this article, we analyzed the ability of *S. suis* and GBS CPSs to activate DCs, as determined by production of cytokines and chemokines susceptible to be involved in TI responses. Mouse-origin DCs were used as mouse models are well described for both pathogens and *in vitro* interactions of GBS or *S. suis* with mouse, human and/or swine origin cells showed similar results (12, 14, 15, 52, 53, 70, 71). We used native and desialylated CPSs isolated from two of the most virulent and frequently isolated serotypes in humans for each bacterial species, in order to examine the role of CPS composition in modulation of DC activation. The capsular preparations underwent a series of rigorous physicochemical and immunologic control quality tests, which attested the high purity of the CPSs, the preservation of epitope recognition, as well as the absence of sialic acid in the desialylated preparations. Our results demonstrated that native *S. suis* types 2, 14 and GBS types III and V CPSs do not induce the release of key pro-inflammatory cytokines, which confirms the poor immunogenic nature of these molecules. This is in accordance with previous experiments where *S. suis* type 2 CPS did not induce the production of either IL-1β, IL-6 or TNF-α by human monocytes or murine macrophages (52, 72). However, our results are in contradiction with earlier studies which showed that GBS type III CPS stimulated the production of TNF-α and IL-6 by human cord blood monocytes (73, 74). This difference could be explained by the use of different cells or by variations in the purification method. Nevertheless, the authors indicated that GBS type III CPS was a poor stimulator compared to other bacterial cell wall components (73, 74). Other studies using CPS purified from other bacterial species have concluded likewise that such a molecule was a poor activator of the immune system. *S. pneumoniae* CPS incubated with human monocyte-derived DCs produced no or very small amounts of IL-12 or IL-10 (75). Similarly, *N. meningitidis* type C CPS was unable to induce production of IL-6 and TNF-α by human monocytes or macrophages (76). By using desialylated CPSs, we demonstrated for the first time that sialic acid does not seem to play an inhibitory role in the release of pro-inflammatory cytokines by DCs. The presence of sialic acid has been linked to production of the regulatory cytokine IL-10 (77, 78). However, sialic acid in either GBS CPSs or *S. suis* CPSs did not result in increased IL-10 production by DCs. Thus, an imbalance between pro- and anti-inflammatory cytokine profiles cannot explain the poorly DC-stimulatory properties of either GBS or *S. suis* CPSs.

Interestingly, *S. suis* and GBS CPSs remarkably stimulated DC production of two members of the CC family of chemokines, CCL2 and CCL3. These two chemokines are known to play a major role in the selective recruitment of monocytes, macrophages, DCs and lymphocytes to sites of inflammation (79, 80). High levels of CCL2 in the central nervous system is a characteristic of patients with bacterial meningitis (81). Systemic production of this chemokine, as well as its expression in the brain, are features of *S. suis* type 2 infected mice (82), and CCL2 has been associated to clinical signs of GBS sepsis in neonates (83). Recently, whole *S. suis* type 2 has been shown to induce CCL2 production by murine DCs (12) and CCL3 production by total mouse splenocytes (unpublished data). Similarly, studies have shown that whole GBS type III stimulates CCL2 and CCL3 secretion by murine macrophages and/or DCs (84, 85). Our observations with purified CPSs allow a better interpretation of previous data showing different patterns of chemokine production obtained with total leukocytes, monocytes and/or DCs cultured in presence of whole *S. suis* type 2 or GBS types III and V, and their respective non-encapsulated mutants. Indeed, in these studies, production of CCL2 and/or CCL3 was significantly diminished with bacteria lacking CPS (52, 71, 85), suggesting an important role of CPS in contributing to the production of these chemokines. Our observations are analog with antecedent studies where *S. suis* type 2 CPS was able to induce the expression of CCL2 mRNA in a porcine whole-blood culture system and the release of this chemokine by human monocytes and murine macrophages (52, 71). Other studies have reported that purified CPSs from *N. meningitidis*, *Porphyromonas gingivalis* and *Bacteroides fragilis* stimulate liberation of chemokines by murine macrophages or DCs (25-27). Surprisingly, differences in composition or structure between *S. suis* and GBS CPSs, or presence of sialic acid, did not deeply influence the release of chemokine by DCs.

It is well known that PRRs, including TLRs, are involved in the activation of immune cells by encapsulated bacteria and/or their purified CPSs (27, 56, 86). Our results indicates that the production of CCL2 by native or desialylated *S. suis* or GBS CPSs was independent of TLR2 or MyD88-related pathways. Similarly, production of CCL2 by murine macrophages incubated with *S. suis* type 2 CPS has been previously shown to be TLR2- and MyD88-independent (52). Macrophage expression of CCL2 induced by whole GBS type III has also been reported to be TLR2-independent (54).

Albeit CCL2 and CCL3 are two members of the CC family of chemokines presenting similarities in the regulation of their synthesis as well as in their biological functions, CCL3 production induced by *S. suis* or GBS CPSs was significantly diminished with MyD88$^{-/-}$ DCs and partially affected with TLR2$^{-/-}$ DCs. Interestingly, and in contrast to CCL2, macrophage expression of CCL3 induced by whole GBS type III has been reported to be TLR2-dependent (54). Albeit differential expression of these two chemokines has been reported in other systems (87), the underlying regulatory mechanisms are unknown. Nevertheless, the partial inhibition of CCL3 production in TLR2$^{-/-}$ or MyD88$^{-/-}$ DCs suggests that other TLRs as well as MyD88-independent pathways may be implicated in chemokine release by *S. suis* or GBS CPSs. The concept of direct interaction of CPS with TLRs is still controversial. Albeit we used highly purified CPS material undergoing strict quality controls, undetected contamination traces of lipoproteins cannot be excluded. Cell deficient in TLR2 might also fail to express adequate levels of a receptor that may be relevant for CPS recognition. Members of the large family of lectin receptors are other possible receptor candidates (77, 88), which warrants further investigations.

CPS antigens are, with few exceptions, considered as TI antigens. It is well known that the TNF family member BAFF plays a crucial role in the immune response against these antigens. Whereas several studies have shown that TLR ligand LPS, or prototype TI antigen NP-Ficoll, stimulate expression or production of BAFF by total splenocytes, macrophages or DCs (29, 57, 59, 89), there is a relative paucity of evidence concerning BAFF induction by CPSs. In our study, we observed that *S. suis* and GBS CPSs were unable to induce a significant expression of BAFF by DCs in comparison with unstimulated cells. This is in accordance with a previous study where *N. meningitidis* type C and GBS type V CPSs did not promote the release of BAFF by murine DCs (57), and confirms the poor immunogenicity of CPSs. We observed that CPSs presented a suppressive effect on the capacity of IFN-γ to induce BAFF expression, and some CPSs were able to provoke an inhibition of up to 40%. Similarly, *N. meningitidis* type C and GBS type V CPSs have been shown to inhibit intracellular and extracellular levels of IFN-γ-induced BAFF in murine DCs (57). We evaluated for the first time the influence of sialic acid on this effect. However, sialylation did

not play a major role in modulation of BAFF expression. The negative regulatory function of CPSs is not only observed on BAFF mRNA synthesis. The presence of CPS impairs cytokine release by DCs and macrophages activated by *S. suis* (12, 15, 52, 70). The inhibitory effect of CPS is not specific of cytokine expression but affects other cell functions such as the expression of co-stimulatory molecules and major histocompatibility complex class II by DCs (15). *S. suis* type 2 CPS was shown to down-modulate phagocytosis by destabilizing lipid microdomains and inhibiting activation of signaling pathways involved in phagocytosis (90, 91), whereas GBS type III CPS impairs bactericidal functions of neutrophils (77).

In conclusion, we found that highly purified CPSs isolated from two distinct serotypes from two different Gram-positive streptococci, *S. suis* and GBS, were principally poor immunogenic antigens. However, they were able to specifically induce production of CCL2 and CCL3 by DCs. TLR2 and other MyD88-dependent pathways are partially involved in recognition of these CPSs, which might also implicate a more complex crosstalk with other receptors. Interestingly, the effect of CPS composition (including sialic acid) and structure on DC function was less marked than that previously reported on B cell activation (67, 68) or than the observed variations in CPS recognition by specific sera in our dot-ELISA analysis. Thus, DCs seem to recognize and respond to these CPSs in a "pattern" way rather than in a structure-dictated manner, which is in agreement with the role of the innate immune system. Further studies are guaranteed on the impact of DC activation status on B cell responses to these TI antigens.

Acknowledgements

This work was mainly supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a grant to MS (#342150-07) and in part by a Canadian Institutes of Health Research grant and by the Fonds de recherche du Québec – Nature et technologies (FRQNT)–New Initiatives program to MS and MRVC.

References

1. Edwards MS, Baker CJ. Group B streptococcal infections in elderly adults. Clin Infect Dis. 2005;41(6):839-47.

2. Koenig JM, Keenan WJ. Group B streptococcus and early-onset sepsis in the era of maternal prophylaxis. Pediatr Clin North Am. 2009;56(3):689-708, Table of Contents.

3. Blumberg HM, Stephens DS, Modansky M, Erwin M, Elliot J, Facklam RR, et al. Invasive group B streptococcal disease: the emergence of serotype V. J Infect Dis. 1996;173(2):365-73.

4. Gottschalk M, Xu J, Calzas C, Segura M. Streptococcus suis: a new emerging or an old neglected zoonotic pathogen? Future Microbiol.5(3):371-91.

5. Segura M. Streptococcus suis: an emerging human threat. J Infect Dis. 2009;199(1):4-6.

6. Gottschalk M, Segura M, Xu J. *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. Anim Health Res Rev. 2007;8(1):29-45.

7. Kerdsin A, Oishi K, Sripakdee S, Boonkerd N, Polwichai P, Nakamura S, et al. Clonal dissemination of human isolates of *Streptococcus suis* serotype 14 in Thailand. J Med Microbiol. 2009;58(Pt 11):1508-13.

8. Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, et al. Structural and genetic diversity of group B streptococcus capsular polysaccharides. Infect Immun. 2005;73(5):3096-103.

9. Fittipaldi N, Segura M, Grenier D, Gottschalk M. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent Streptococcus suis. Future Microbiol.7(2):259-79.

10. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of Streptococcus suis serotype 2 capsular polysaccharide. Biochem Cell Biol.88(3):513-25.

11. Van Calsteren MR, C. Calzas, G. Goyette-Desjardins, M. Okura, D. Takamatsu, M. Gottschalk, and M. Segura. *Structure determination of Streptococcus suis serotype 14 capsular polysaccharide*. Biochem Cell Biol. 2012;In press.

12. Lecours MP, Gottschalk M, Houde M, Lemire P, Fittipaldi N, Segura M. Critical role for Streptococcus suis cell wall modifications and suilysin in resistance to complement-dependent killing by dendritic cells. J Infect Dis.204(6):919-29.

13. Lemire P, Houde M, Lecours MP, Fittipaldi N, Segura M. Role of capsular polysaccharide in Group B Streptococccus interactions with dendritic cells. Microbes Infect.

14. Segura MA, Cleroux P, Gottschalk M. Streptococcus suis and group B Streptococcus differ in their interactions with murine macrophages. FEMS Immunol Med Microbiol. 1998;21(3):189-95.

15. Lecours MP, Segura M, Lachance C, Mussa T, Surprenant C, Montoya M, et al. Characterization of porcine dendritic cell response to Streptococcus suis. Vet Res.42(1):72.

16. Takahashi S, Aoyagi Y, Adderson EE, Okuwaki Y, Bohnsack JF. Capsular sialic acid limits C5a production on type III group B streptococci. Infect Immun. 1999;67(4):1866-70.

17. Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. Nat Rev Immunol. 2007;7(4):255-66.

18. Chaffin DO, Mentele LM, Rubens CE. Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. J Bacteriol. 2005;187(13):4615-26.

19. Lecours MP, Fittipaldi N, Takamatsu D, Okura M, Segura M, Goyette-Desjardins G, et al. Sialylation of Streptococcus suis serotype 2 is essential for capsule expression but is not responsible for the main capsular epitope. Microbes Infect.

20. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell.140(6):805-20.

21. Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. 1991;9:271-96.

22. Mond JJ, Vos Q, Lees A, Snapper CM. T cell independent antigens. Curr Opin Immunol. 1995;7(3):349-54.

23. Weintraub A. Immunology of bacterial polysaccharide antigens. Carbohydr Res. 2003;338(23):2539-47.

24. Simpson SQ, Singh R, Bice DE. Heat-killed pneumococci and pneumococcal capsular polysaccharides stimulate tumor necrosis factor-alpha production by murine macrophages. Am J Respir Cell Mol Biol. 1994;10(3):284-9.

25. Wang Q, McLoughlin RM, Cobb BA, Charrel-Dennis M, Zaleski KJ, Golenbock D, et al. A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2. J Exp Med. 2006;203(13):2853-63.

26. d'Empaire G, Baer MT, Gibson FC, 3rd. The K1 serotype capsular polysaccharide of Porphyromonas gingivalis elicits chemokine production from murine macrophages that facilitates cell migration. Infect Immun. 2006;74(11):6236-43.

27. Zughaier SM. Neisseria meningitidis capsular polysaccharides induce inflammatory responses via TLR2 and TLR4-MD-2. J Leukoc Biol.89(3):469-80.

28. Soell M, Diab M, Haan-Archipoff G, Beretz A, Herbelin C, Poutrel B, et al. Capsular polysaccharide types 5 and 8 of Staphylococcus aureus bind specifically to human epithelial (KB) cells, endothelial cells, and monocytes and induce release of cytokines. Infect Immun. 1995;63(4):1380-6.

29. Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol. 2002;3(9):822-9.

30. Craxton A, Magaletti D, Ryan EJ, Clark EA. Macrophage- and dendritic cell--dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. Blood. 2003;101(11):4464-71.

31. Jacques M, Gottschalk M, Foiry B, Higgins R. Ultrastructural study of surface components of Streptococcus suis. J Bacteriol. 1990;172(6):2833-8.

32. Gottschalk M, Higgins R, Jacques M, Mittal KR, Henrichsen J. Description of 14 new capsular types of *Streptococcus suis*. J Clin Microbiol. 1989;27(12):2633-6.

33. Kuypers JM, Heggen LM, Rubens CE. Molecular analysis of a region of the group B streptococcus chromosome involved in type III capsule expression. Infect Immun. 1989;57(10):3058-65.

34. Michon F. Procedures for the extraction and isolation of bacterial capsular polysaccharides for use as vaccines or linked to proteins as conjugate vaccines. US patent 6,248,570 B1. June 2001.

35. Brandrup J, E. H. Immergut, and E. A. Grulke. Polymer handbook, 4th ed. John Wiley & Sons, New York, NY. 2005.

36. Gornik O, Lauc G. Enzyme linked lectin assay (ELLA) for direct analysis of transferrin sialylation in serum samples. Clin Biochem. 2007;40(9-10):718-23.

37. Shibuya N, Goldstein IJ, Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ. The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. J Biol Chem. 1987;262(4):1596-601.

38. Geisler C, Jarvis DL. Effective glycoanalysis with Maackia amurensis lectins requires a clear understanding of their binding specificities. Glycobiology.21(8):988-93.

39. Miller RL, Collawn JF, Jr., Fish WW. Purification and macromolecular properties of a sialic acid-specific lectin from the slug Limax flavus. J Biol Chem. 1982;257(13):7574-80.

40. Charland N, Jacques M, Lacouture S, Gottschalk M. Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by Streptococcus suis serotypes 1, 2 and 1/2. Microbiology. 1997;143 (Pt 11):3607-14.

41. Higgins R, Gottschalk M. An update on *Streptococcus suis* identification. J Vet Diagn Invest. 1990;2(3):249-52.

42. Segura M, Su Z, Piccirillo C, Stevenson MM. Impairment of dendritic cell function by excretory-secretory products: a potential mechanism for nematode-induced immunosuppression. Eur J Immunol. 2007;37(7):1887-904.

43. van den Berg TK, Kraal G. A function for the macrophage F4/80 molecule in tolerance induction. Trends Immunol. 2005;26(10):506-9.

44. MacDonald AS, Straw AD, Bauman B, Pearce EJ. CD8- dendritic cell activation status plays an integral role in influencing Th2 response development. J Immunol. 2001;167(4):1982-8.

45. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods. 1999;223(1):77-92.

46. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.

47. Deng L, Kasper DL, Krick TP, Wessels MR. Characterization of the linkage between the type III capsular polysaccharide and the bacterial cell wall of group B Streptococcus. J Biol Chem. 2000;275(11):7497-504.

48. Gonzalez-Outeirino J, Kadirvelraj R, Woods RJ. Structural elucidation of type III group B Streptococcus capsular polysaccharide using molecular dynamics simulations: the role of sialic acid. Carbohydr Res. 2005;340(5):1007-18.

49. Guttormsen HK, Paoletti LC, Mansfield KG, Jachymek W, Jennings HJ, Kasper DL. Rational chemical design of the carbohydrate in a glycoconjugate vaccine enhances IgM-to-IgG switching. Proc Natl Acad Sci U S A. 2008;105(15):5903-8.

50. Jennings HJ, Lugowski C, Kasper DL. Conformational aspects critical to the immunospecificity of the type III Group B streptococcal polysaccharide. Biochemistry. 1981;20(16):4511-8.

51. Knibbs RN, Goldstein IJ, Ratcliffe RM, Shibuya N. Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from Maackia amurensis. Comparison with other sialic acid-specific lectins. J Biol Chem. 1991;266(1):83-8.

52. Graveline R, Segura M, Radzioch D, Gottschalk M. TLR2-dependent recognition of Streptococcus suis is modulated by the presence of capsular polysaccharide which modifies macrophage responsiveness. Int Immunol. 2007;19(4):375-89.

53. Lecours MP, Segura M, Fittipaldi N, Rivest S, Gottschalk M. Immune Receptors Involved in Streptococcus suis Recognition by Dendritic Cells. PLoS One.7(9):e44746.

54. Draper DW, Bethea HN, He YW. Toll-like receptor 2-dependent and -independent activation of macrophages by group B streptococci. Immunol Lett. 2006;102(2):202-14.

55. Henneke P, Takeuchi O, van Strijp JA, Guttormsen HK, Smith JA, Schromm AB, et al. Novel engagement of CD14 and multiple toll-like receptors by group B streptococci. J Immunol. 2001;167(12):7069-76.

56. Um SH, Rhee DK, Pyo S. Involvement of protein kinase C and tyrosin kinase in tumoricidal activation of macrophage induced by Streptococcus pneumoniae type II capsular polysaccharide. Int Immunopharmacol. 2002;2(1):129-37.

57. Kanswal S, Katsenelson N, Allman W, Uslu K, Blake MS, Akkoyunlu M. Suppressive effect of bacterial polysaccharides on BAFF system is responsible for their poor immunogenicity. J Immunol.186(4):2430-43.

58. Kim HA, Jeon SH, Seo GY, Park JB, Kim PH. TGF-beta1 and IFN-gamma stimulate mouse macrophages to express BAFF via different signaling pathways. J Leukoc Biol. 2008;83(6):1431-9.

59. Nardelli B, Belvedere O, Roschke V, Moore PA, Olsen HS, Migone TS, et al. Synthesis and release of B-lymphocyte stimulator from myeloid cells. Blood. 2001;97(1):198-204.

60. Courtney AH, Puffer EB, Pontrello JK, Yang ZQ, Kiessling LL. Sialylated multivalent antigens engage CD22 in trans and inhibit B cell activation. Proc Natl Acad Sci U S A. 2009;106(8):2500-5.

61. Elliott SD, Clifton-Hadley F, Tai J. Streptococcal infection in young pigs. V. An immunogenic polysaccharide from *Streptococcus suis* type 2 with particular reference to vaccination against streptococcal meningitis in pigs. J Hyg (Lond). 1980;85(2):275-85.

62. Baums CG, Kock C, Beineke A, Bennecke K, Goethe R, Schroder C, et al. Streptococcus suis bacterin and subunit vaccine immunogenicities and protective efficacies against serotypes 2 and 9. Clin Vaccine Immunol. 2009;16(2):200-8.

63. del Campo Sepulveda EM, Altman E, Kobisch M, D'Allaire S, Gottschalk M. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect ELISA. Vet Microbiol. 1996;52(1-2):113-25.

64. Heath PT. An update on vaccination against group B streptococcus. Expert Rev Vaccines.10(5):685-94.

65. Baker CJ, Kasper DL. Correlation of maternal antibody deficiency with susceptibility to neonatal group B streptococcal infection. N Engl J Med. 1976;294(14):753-6.

66. Avci FY, Kasper DL. How bacterial carbohydrates influence the adaptive immune system. Annu Rev Immunol.28:107-30.

67. Wessels MR, Paoletti LC, Rodewald AK, Michon F, DiFabio J, Jennings HJ, et al. Stimulation of protective antibodies against type Ia and Ib Group B *Streptococci* by a type Ia polysaccharide-tetanus toxoid conjugate vaccine. Infect Immun. 1993;61(11):4760-6.

68. Finne J, Leinonen M, Makela PH. Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. Lancet. 1983;2(8346):355-7.

69. Moe GR, Bhandari TS, Flitter BA. Vaccines containing de-N-acetyl sialic acid elicit antibodies protective against neisseria meningitidis groups B and C. J Immunol. 2009;182(10):6610-7.

70. Meijerink M, Ferrando ML, Lammers G, Taverne N, Smith HE, Wells JM. Immunomodulatory effects of Streptococcus suis capsule type on human dendritic cell responses, phagocytosis and intracellular survival. PLoS One.7(4):e35849.

71. Segura M, Vanier G, Al-Numani D, Lacouture S, Olivier M, Gottschalk M. Proinflammatory cytokine and chemokine modulation by Streptococcus suis in a whole-blood culture system. FEMS Immunol Med Microbiol. 2006;47(1):92-106.

72. Segura M, Stankova J, Gottschalk M. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect Immun. 1999;67(9):4646-54.

73. Vallejo JG, Baker CJ, Edwards MS. Roles of the bacterial cell wall and capsule in induction of tumor necrosis factor alpha by type III group B streptococci. Infect Immun. 1996;64(12):5042-6.

74. Vallejo JG, Baker CJ, Edwards MS. Interleukin-6 production by human neonatal monocytes stimulated by type III group B streptococci. J Infect Dis. 1996;174(2):332-7.

75. Meltzer U, Goldblatt D. Pneumococcal polysaccharides interact with human dendritic cells. Infect Immun. 2006;74(3):1890-5.

76. Kocabas C, Katsenelson N, Kanswal S, Kennedy MN, Cui X, Blake MS, et al. Neisseria meningitidis type C capsular polysaccharide inhibits lipooligosaccharide-induced cell activation by binding to CD14. Cell Microbiol. 2007;9(5):1297-310.

77. Carlin AF, Uchiyama S, Chang YC, Lewis AL, Nizet V, Varki A. Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response. Blood. 2009;113(14):3333-6.

78. Ando M, Tu W, Nishijima K, Iijima S. Siglec-9 enhances IL-10 production in macrophages via tyrosine-based motifs. Biochem Biophys Res Commun. 2008;369(3):878-83.

79. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009;29(6):313-26.

80. Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol. 2004;36(10):1882-6.

81. Lahrtz F, Piali L, Spanaus KS, Seebach J, Fontana A. Chemokines and chemotaxis of leukocytes in infectious meningitis. J Neuroimmunol. 1998;85(1):33-43.

82. Dominguez-Punaro MC, Segura M, Plante MM, Lacouture S, Rivest S, Gottschalk M. *Streptococcus suis* serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection. J Immunol. 2007;179(3):1842-54.

83. Okazaki K, Kondo M, Kato M, Nishida A, Takahashi H, Noda M, et al. Temporal alterations in concentrations of sera cytokines/chemokines in sepsis due to group B streptococcus infection in a neonate. Jpn J Infect Dis. 2008;61(5):382-5.

84. Fan H, Williams DL, Zingarelli B, Breuel KF, Teti G, Tempel GE, et al. Differential regulation of lipopolysaccharide and Gram-positive bacteria induced cytokine and chemokine production in macrophages by Galpha(i) proteins. Immunology. 2007;122(1):116-23.

85. Lemire P, Houde M, Segura M. Encapsulated group B Streptococcus modulates dendritic cell functions via lipid rafts and clathrin-mediated endocytosis. Cell Microbiol.

86. Mogensen TH, Paludan SR, Kilian M, Ostergaard L. Live Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. J Leukoc Biol. 2006;80(2):267-77.

87. Mitchell D, Olive C. Regulation of Toll-like receptor-induced chemokine production in murine dendritic cells by mitogen-activated protein kinases. Mol Immunol.47(11-12):2065-73.

88. Zamze S, Martinez-Pomares L, Jones H, Taylor PR, Stillion RJ, Gordon S, et al. Recognition of bacterial capsular polysaccharides and lipopolysaccharides by the macrophage mannose receptor. J Biol Chem. 2002;277(44):41613-23.

89. Moon EY, Lee JH, Oh SY, Ryu SK, Kim HM, Kwak HS, et al. Reactive oxygen species augment B-cell-activating factor expression. Free Radic Biol Med. 2006;40(12):2103-11.

90. Houde M, Gottschalk M, Gagnon F, Van Calsteren M-R, Segura M. *Streptococcus suis* Capsular Polysaccharide Inhibits Phagocytosis through Destabilization of Lipid Microdomains and Prevents Lactosylceramide-Dependent Recognition. Infect Immun. 2012;80(2):506-17.

91. Segura M, Gottschalk M, Olivier M. Encapsulated Streptococcus suis inhibits activation of signaling pathways involved in phagocytosis. Infect Immun. 2004;72(9):5322-30.

Tables

Table 1. Quality control tests of purified *S. suis* and GBS capsular polysaccharides (CPSs).

CPS	Sugar composition[a]	M_w[b] (kDa)	Nucleic acid[c] (%)	Protein[d] (%)
S. suis 2 (n)	1:2.6:0.9:1.0:0.9	480	0.8	n.s.
S. suis 2 (dS)		21		
S. suis 14 (n)	1:2.5:0.8:1.0:0.0	500	0.7	n.s.
S. suis 14 (dS)		176		
GBS III (n)	1:1.5:1.0:0.9:0.0	108	0.8	n.s.
GBS III (dS)		59		
GBS V (n)	3:1.5:0.8:0.8:0.0	128	0.9	n.s.
GBS V (dS)		91		

[a] Determined by gas chromatography (GC) after methanolysis and acetylation (in sugar order: glucose:galactose:*N*-acetylglucosamine:*N*-acetylneuraminic acid:rhamnose).

[b] Determined by size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS).

[c] Percentage (w/w) nucleic acid determined by spectrophotometry at 230 and 260 nm.

[d] Percentage (w/w) protein determined by spectrophotometry at 750 nm.

n.s.: non significant, under the limit of detection

M_w: weight-average molecular mass; n: native; dS: desialylated

See Materials and Methods section for more details.

Figures

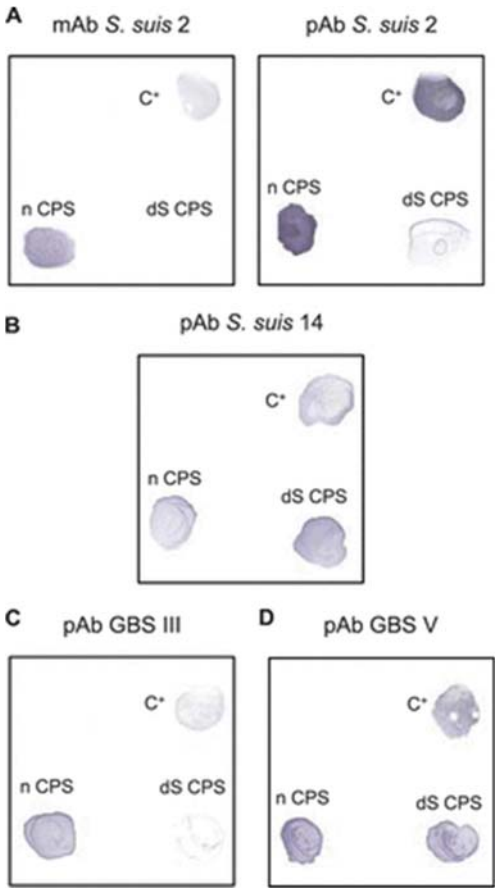


Figure 1. Recognition of *S. suis* and GBS capsular polysaccharides (CPSs) by dot-ELISA.

S. suis 2 (A), *S. suis* 14 (B), GBS III (C) or GBS V (D) whole bacteria (positive control C^+) (10^7 CFU) or their respective purified native (n) or desialylated (dS) CPS (10 µg) were incubated with a monoclonal antibody (mAb) directed against the sialic acid moiety of *S. suis* 2 CPS (A, left) or with mono-specific polyclonal sera (pAb) against *S. suis* 2 (A, right), *S. suis* 14 (B), GBS III CPS (C) or GBS V CPS (D).

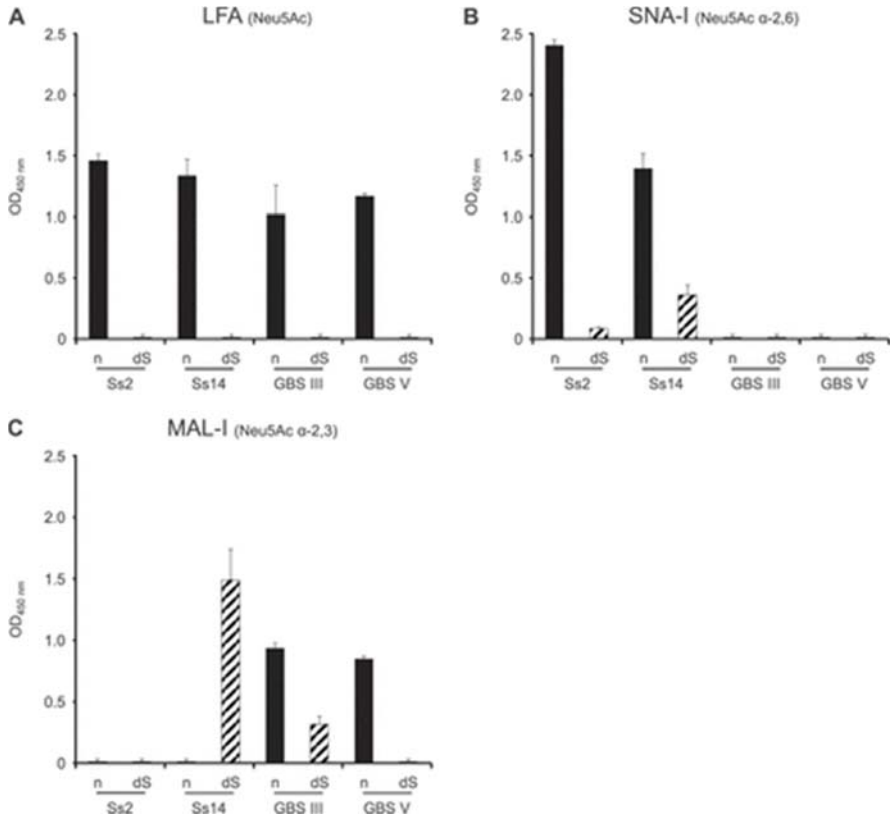


Figure 2. Recognition of *S. suis* and GBS capsular polysaccharides (CPSs) by enzyme-linked lectin assay (ELLA).

Native (n) or desialylated (dS) *S. suis* 2 (Ss2), *S. suis* 14 (Ss14), GBS III or GBS V CPS (each at 2 μg/ml) was incubated with *Limax flavus* agglutinin specific for Neu5Ac (LFA) (A), *Sambucus nigra* agglutinin specific for Neu5Ac α-2,6 link (SNA-I) (B) or *Maackia amurensis* leukoagglutinin specific for Neu5Ac α-2,3 link (MAL-I) (C). Data are expressed as mean ± SEM (optical density $OD_{450\ nm}$) from at least three experiments with at least three technical replicates and are corrected for reaction of dilution buffer with the corresponding lectin.

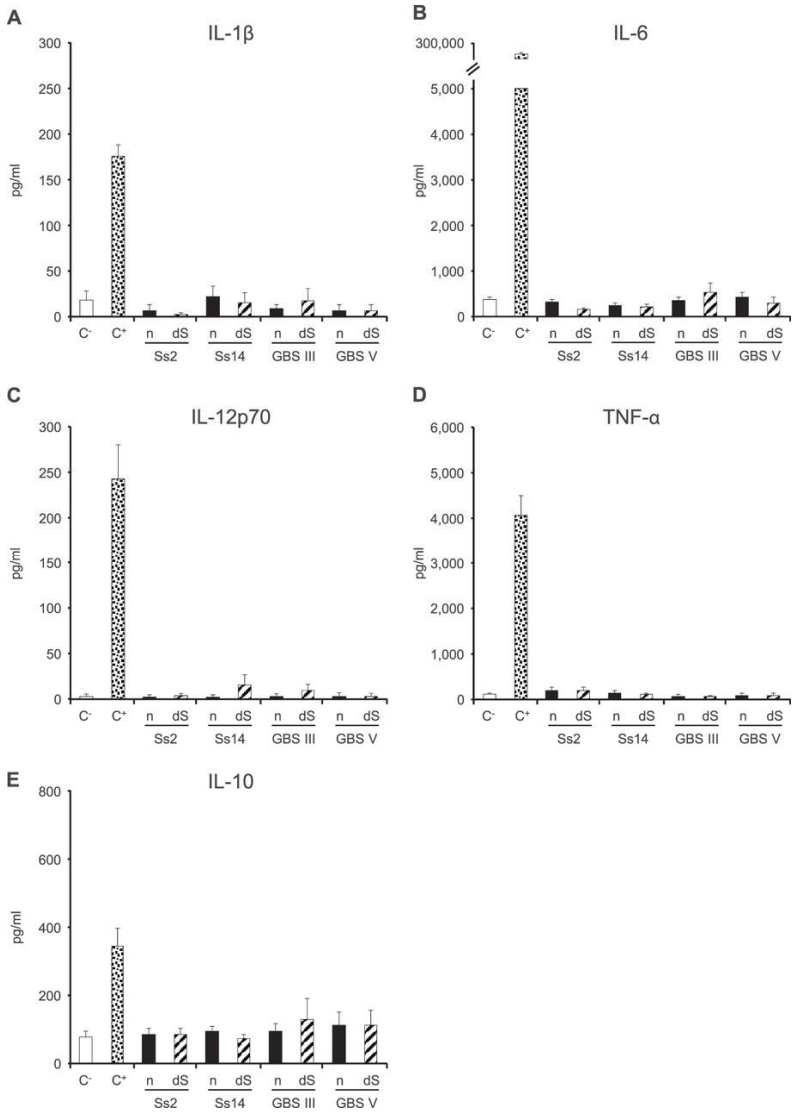


Figure 3. Pro- and anti-inflammatory cytokine production by DCs in response to stimulation by *S. suis* or GBS capsular polysaccharides (CPSs) for 24 h.

Native (n) or desialylated (dS) *S. suis* 2 (Ss2), *S. suis* 14 (Ss14), GBS III or GBS V CPS (each at 200 µg/ml) was incubated with DCs (10^6 cells/ml). After 24 h, supernatants were collected and IL-1β (A), IL-6 (B), IL-12p70 (C), TNF-α (D) and IL-10 (E) levels were quantified by ELISA. Cells stimulated with medium alone or LPS (1 µg/ml) served as negative (C^-) and positive (C^+) control, respectively. Data are expressed as mean ± SEM (pg/ml) from at least three experiments with at least three technical replicates.

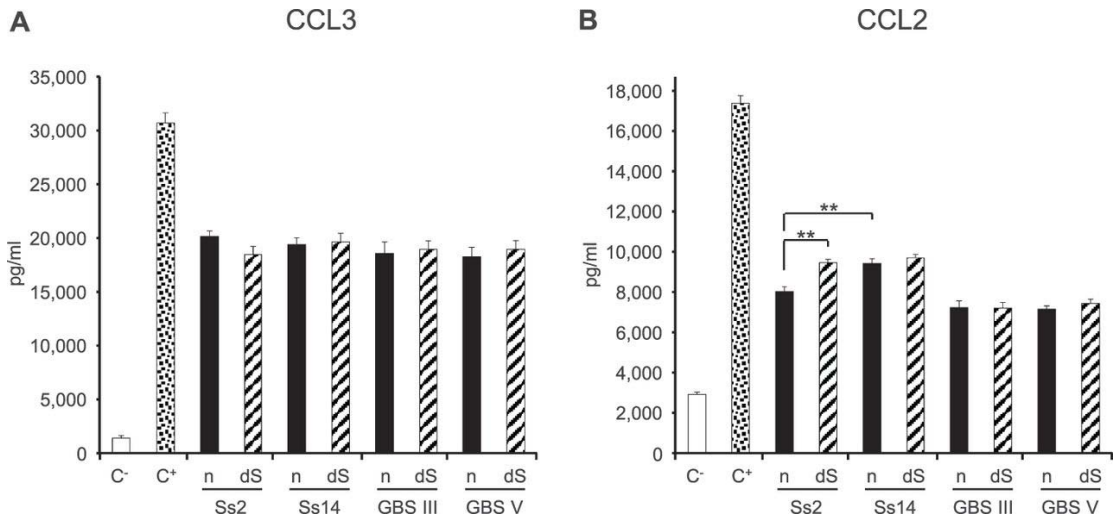


Figure 4. Chemokine production by DCs in response to stimulation by *S. suis* or GBS capsular polysaccharides (CPSs) for 24 h.

Native (n) or desialylated (dS) *S. suis* 2 (Ss2), *S. suis* 14 (Ss14), GBS III or GBS V CPS (each at 200 µg/ml) was incubated with DCs (10^6 cells/ml). After 24 h, supernatants were collected and CCL3 (A) and CCL2 (B) levels were quantified by ELISA. Cells stimulated with medium alone or LPS (1 µg/ml) served as negative (C^-) and positive (C^+) control, respectively. Data are expressed as mean ± SEM (pg/ml) from at least three experiments with at least three technical replicates. **, $P < 0.001$.

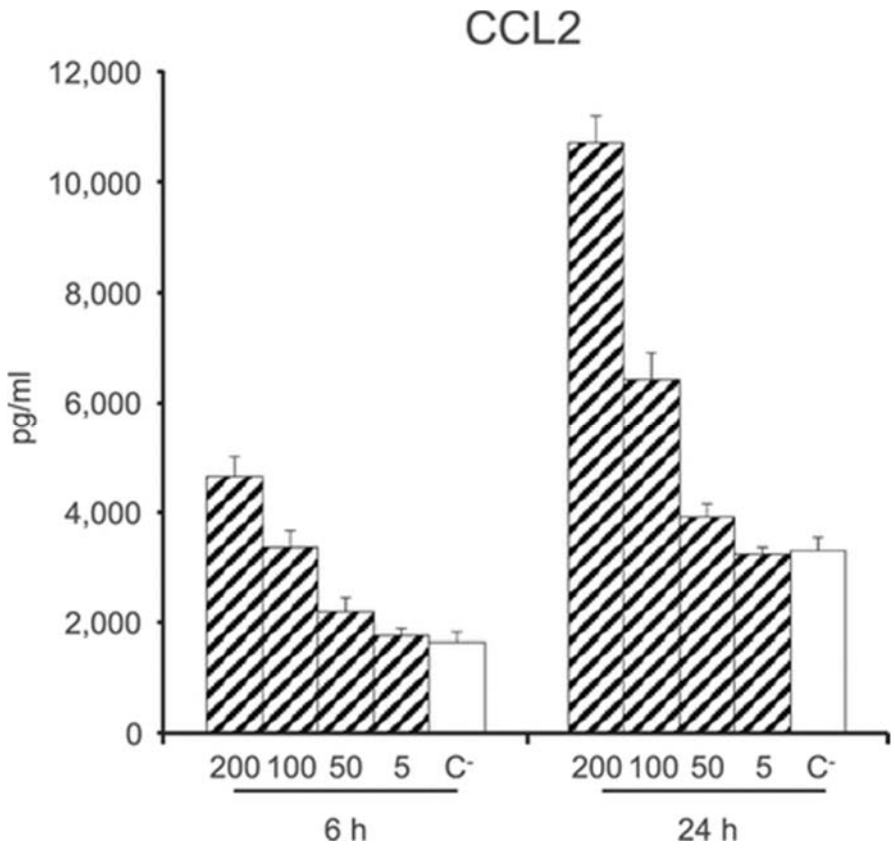


Figure 5. CCL2 production by DCs in response to stimulation by *S. suis* 2 capsular polysaccharide (CPS) is dose and time dependent.

Native *S. suis* 2 CPS (200, 100, 50, 5 µg/ml) was incubated with DCs (10^6 cells/ml). After 6 or 24 h, supernatants were collected and CCL2 levels were quantified by ELISA. Cells stimulated with medium alone served as negative control (C^-). Data are expressed as mean ± SEM (pg/ml) from at least three experiments with at least three technical replicates.

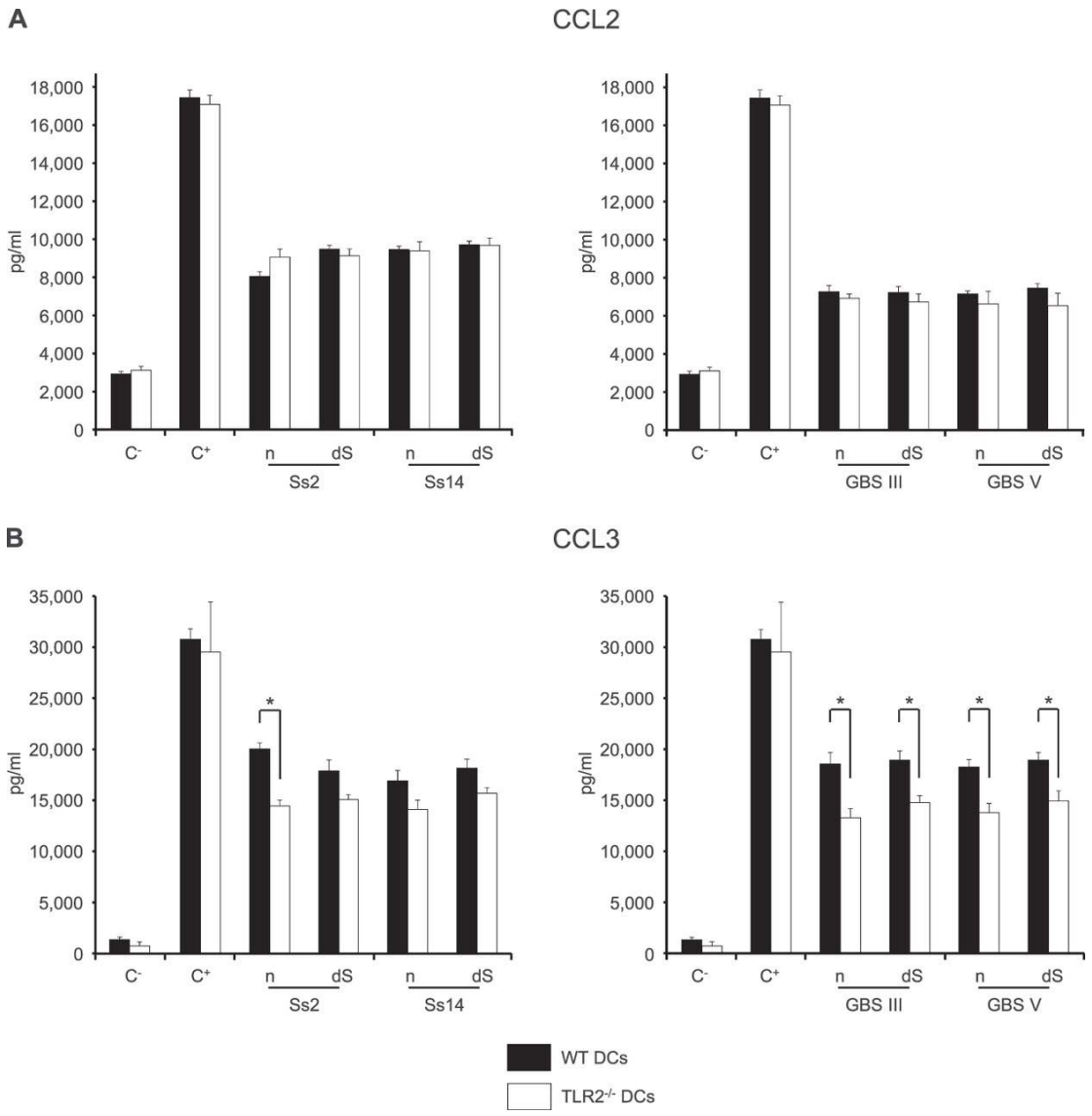


Figure 6. Role of TLR2 in chemokine production by DCs in response to stimulation by *S. suis* or GBS capsular polysaccharides (CPSs) for 24 h.

Native (n) or desialylated (dS) *S. suis* 2 (Ss2), *S. suis* 14 (Ss14), GBS III or GBS V CPS (each at 200 µg/ml) was incubated with WT (black bars) or TLR2$^{-/-}$ (white bars) DCs (10^6 cells/ml). After 24 h, supernatants were collected and CCL2 (A) and CCL3 (B) levels were quantified by ELISA. Cells stimulated with medium alone or LPS (1 µg/ml) served as negative (C^-) and positive (C^+) control, respectively. Data are expressed as mean ± SEM (pg/ml) from at least three experiments with at least three technical replicates. *, $P < 0.05$.

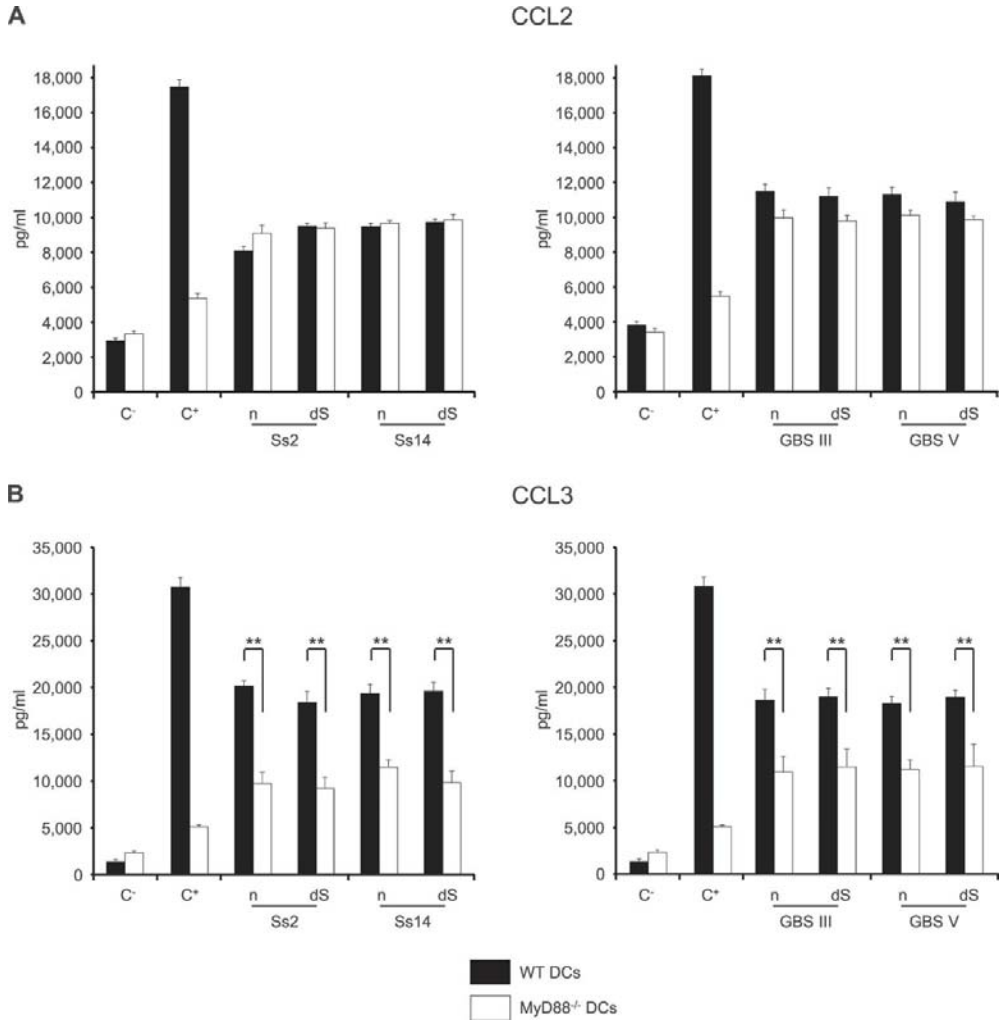


Figure 7. Role of MyD88 in chemokine production by DCs in response to stimulation by *S. suis* or GBS capsular polysaccharides (CPSs) for 24 h.

Native (n) or desialylated (dS) *S. suis* 2 (Ss2), *S. suis* 14 (Ss14), GBS III or GBS V CPS (each at 200 µg/ml) was incubated with WT (black bars) or MyD88$^{-/-}$ (white bars) DCs (10^6 cells/ml). After 24 h, supernatants were collected and CCL2 (A) and CCL3 (B) levels were quantified by ELISA. Cells stimulated with medium alone or LPS (1 µg/ml) served as negative (C^-) and positive (C^+) control, respectively. Data are expressed as mean ± SEM (pg/ml) from at least three experiments with at least three technical replicates. **, $P < 0.001$.

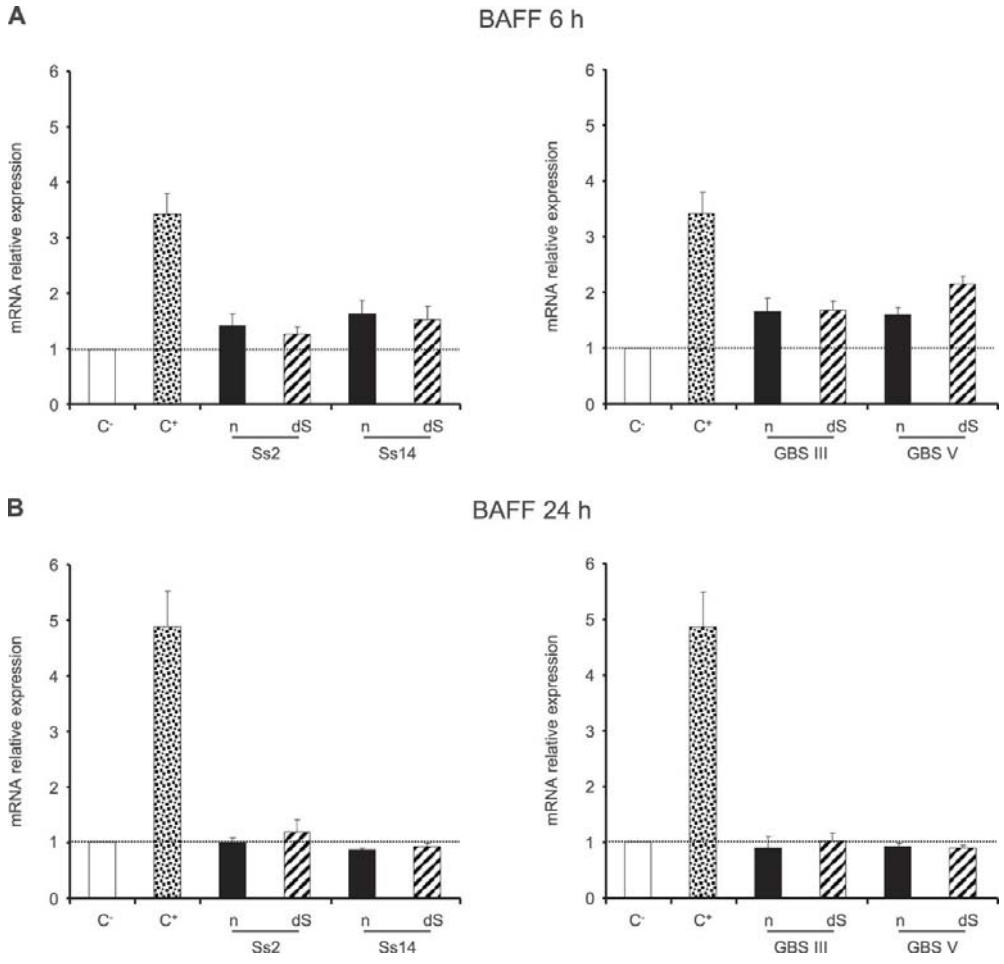


Figure 8. Relative expression of BAFF mRNA by DCs in response to stimulation by *S. suis* or GBS capsular polysaccharides (CPSs) for 24 h.

Native (n) or desialylated (dS) *S. suis* 2 (Ss2), *S. suis* 14 (Ss14), GBS III or GBS V CPS (each at 200 µg/ml) was incubated with DCs (10^6 cells/ml). After 6 (A) or 24 h (B) of incubation, cells were collected and BAFF mRNA expression was determined by RT-qPCR. Cells stimulated with IFN-γ (10 ng/ml) served as positive control (C^+). Data are expressed as mean ± SEM from at least three experiments and are relative to DCs stimulated with medium alone (C^-) whose value was arbitrarily fixed to 1.

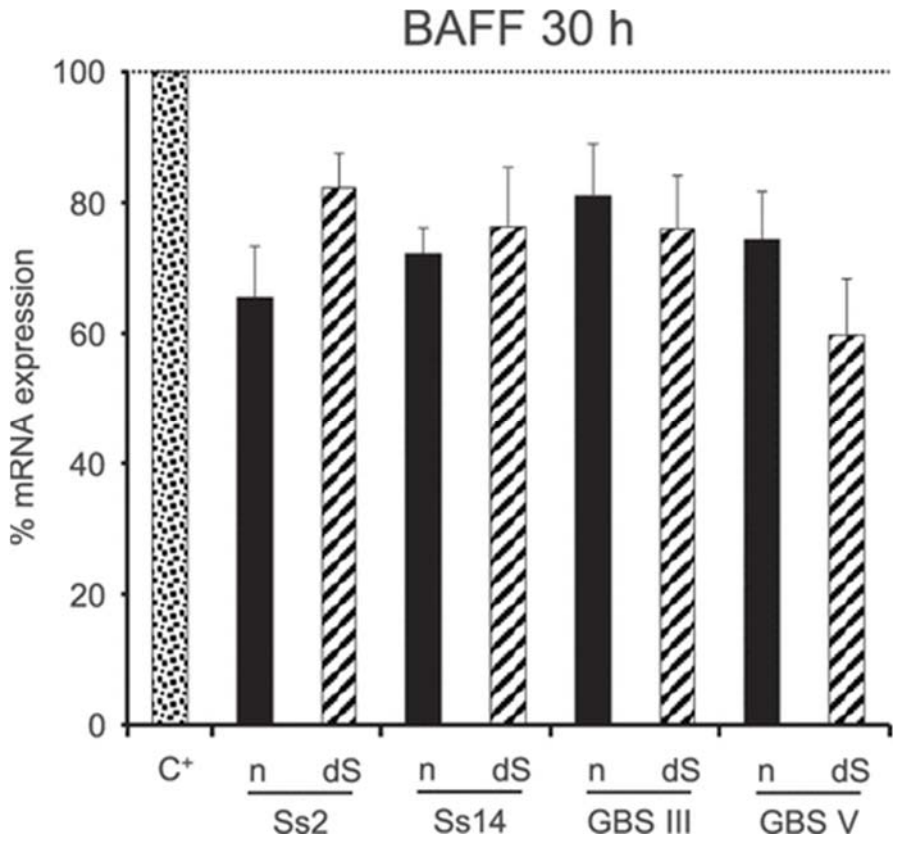


Figure 9. Effect of pre-incubation of DCs with *S. suis* or GBS capsular polysaccharides (CPSs) on IFN-γ-induced expression of BAFF mRNA.

DCs (10^6 cells/ml) were pre-stimulated with native (n) or desialylated (dS) *S. suis* 2 (Ss2), *S. suis* 14 (Ss14), GBS III or GBS V CPS (each at 200 µg/ml) for 6 h prior to incubation with IFN-γ (10 ng/ml) for 24 h. Cells were then collected and BAFF mRNA expression was determined by RT-qPCR. Cells pre-stimulated with medium alone before addition of IFN-γ served as positive control (C^+). Data are expressed as mean ± SEM from at least three experiments and are relative to C^+ whose value was arbitrarily fixed to 100%.

Supporting information

Supplementary material is available with the article through the journal Web site at https://iai.asm.org/content/81/9/3106.

Fig. S1. Nuclear magnetic resonance spectra of native and desialylated S. suis capsular polysaccharides.

Fig. S2. Nuclear magnetic resonance spectra of native and desialylated GBS capsular polysaccharides.

Table S1. Sequences of murine-specific real-time PCR primers.

ANNEXES - ARTICLE XVI

Porcine Dendritic Cells as an In Vitro Model to Assess the Immunological Behaviour of *Streptococcus suis* Subunit Vaccine Formulations and the Polarizing Effect of Adjuvants

---

Léa Martelet [1], Sonia Lacouture [2], **Guillaume Goyette-Desjardins** [1], Guy Beauchamp [3], Charles Surprenant [4], Marcelo Gottschalk [2] and Mariela Segura [1]

[1] Laboratory of Immunology of the Swine and Poultry Infectious Diseases Research Centre (CRIPA), Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC J2S 2M2, Canada

[2] Laboratory of *Streptococcus suis*, CRIPA, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC J2S 2M2, Canada

[3] Biostatistics Office, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC J2S 2M2, Canada

[4] F. Ménard Inc., 251 Route 235, Ange Gardien, QC J0E 1E0, Canada

Publié dans *Pathogens*. **2017**. 6(1):E13.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé partiellement à la réalisation des expériences (préparations de CPS, TT, glycoconjugué CPS-TT, et des adjuvants; 10%).

Abstract

An in vitro porcine bone marrow-derived dendritic cell (DC) culture was developed as a model for evaluating immune polarization induced by adjuvants when administered with immunogens that may become vaccine candidates if appropriately formulated. The swine pathogen *Streptococcus suis* was chosen as a prototype to evaluate proposed *S. suis* vaccine candidates in combination with the adjuvants Poly I:C, Quil A ®, Alhydrogel ®, TiterMax Gold ® and Stimune ®. The toll-like receptor ligand Poly I:C and the saponin Quil A ® polarized swine DC cytokines towards a type 1 phenotype, with preferential production of IL-12 and TNF-α. The water-in-oil adjuvants TiterMax Gold ® and Stimune ® favoured a type 2 profile as suggested by a marked IL-6 release. In contrast, Alhydrogel ® induced a type 1/type 2 mixed cytokine profile. The antigen type differently modified the magnitude of the adjuvant effect, but overall polarization was preserved. This is the first comparative report on swine DC immune activation by different adjuvants. Although further swine immunization studies would be required to better characterize the induced responses, the herein proposed in vitro model is a promising approach that helps assessing behaviour of the vaccine formulation rapidly at the pre-screening stage and will certainly reduce numbers of animals used while advancing vaccinology science.

1. Introduction

Streptococcus suis is an encapsulated bacterium and an important cause of disease in swine, including meningitis, septicemia with sudden death, endocarditis and arthritis. In addition to the important economic losses to the swine industry, *S. suis* is also an emerging zoonotic pathogen (1, 2). *S. suis* was originally classified into 35 serotypes based on the capsular polysaccharide (CPS) antigenicity, although the current taxonomical situation has been recently revised (3). Nevertheless, serotype 2 remains the most virulent and frequent capsular type, isolated worldwide from both swine and humans (4). A high genetic and phenotypic diversity of *S. suis* strains within serotype 2 is reported according to geographical distribution (4). Pigs are affected generally between 5 and 10 weeks of age, when passive immunity provided by colostrum decreases (5, 6). The pathogenesis of *S. suis* infection is not fully understood. In swine, the main port of entry for *S. suis* is the upper respiratory tract (7). Subsequently, this pathogen can overthrow the immune system, through an arsenal of virulence factors, including the CPS (8), to cause acute septicemia that may lead to septic shock or different clinical outcomes depending on the colonized organ via mechanisms that are only partially elucidated to date (9).

So far, bacterins (either commercial or, more commonly, autogenous vaccines) have been used in the field to prevent *S. suis* disease with controversial results, and demonstrating only homologous protection (10). Other strategies, such as live-attenuated or sub-unit vaccines, have been experimentally tested. The use of live avirulent strains gave inconsistent results and may present some safety concerns (zoonosis) (10). More recently, research has focused on *S. suis* surface proteins as potential sub-unit vaccine candidates. One candidate is enolase, a *S. suis* 52-kDa surface protein that it is expressed by *S. suis* of all serotypes and is involved in adhesion to extracellular matrix components. Furthermore, enolase is a highly conserved protein, and anti-enolase antibodies have been detected in convalescent pig sera (11). Nevertheless, immunization studies showed that protection conferred by enolase seems to depend on the adjuvant used in the vaccine formulation (12-14). Similarly, it was recently reported that generation of protective antibodies after immunization with *S. suis* CPS as antigen was restricted to CPS conjugation to an immunogenic carrier protein and the use of emulsifying adjuvants (15). CPS is a promising vaccine candidate because it is the antigen at

the base of serotyping and thus can confer universal protection against all strains within the same serotype (10).

The development of the immune response starts with activation of different types of cells involved in the innate immunity including antigen presenting cells (APCs), such as dendritic cells (DCs). DCs are powerful APCs and strongly influence the outcome of the ensuing immunological events. After the capture of antigens, DCs undergo a maturation process and release several cytokines playing a key role in the development of the adaptive immune response. Indeed, mature DCs migrate to adjacent lymphoid organs where they activate T cells. The action of different cytokine pathways drives differentiation of T cells into distinct subtypes, such as Th1 and Th2, which ultimately influence the class of humoral immune responses elicited by B cells. For example, IL-12 and TNF-α are associated with the production of Th1 cells and type 1 IgG subclasses (IgG2a, IgG2b, IgG2c and IgG3, in mice), whereas IL-6 and other Th2 cytokines contribute to type 2 IgG subclass (IgG1) production (16-18). Indeed, IL-6 promotes IL-4-induced Th2 differentiation and inhibits IL-12-induced Th1 differentiation (19). Different IgG subclasses fulfill distinct biological functions, and thus their relative production is an important consideration when evaluating protection by a vaccine candidate (20, 21). For example, in mice, a dominant type 1 antibody response is associated with opsonophagocytosis and clearance of extracellular encapsulated bacteria, like *S. suis* (10, 22, 23). In pigs, the concept of type 1/type 2 IgG subclasses is not completely documented. Crawley et al. (24) determined that porcine Th1 cytokines, IFN-γ and IL-12, induce an IgG2 profile in pigs and that porcine IgG2 activates complement more efficiently than IgG1. In this regard, recent studies suggested that swine IgG2 antibodies are more capable of opsonization than IgG1 antibodies (10, 25).

Adjuvants can dramatically influence the vaccine-induced antibody response including bias to type 1 or type 2 responses, which may have a significant effect on the protective efficacy of a vaccine (26, 27). For instance, *S. suis* protein candidates may behave as protective (14, 22) or non-protective (12, 28) immunogens at least in part depending on the adjuvant. Compared to human medicine, a wider range of adjuvants has been successfully used in commercial vaccines for animals and several new technologies are currently in

preclinical development (reviewed in (29, 30)). These adjuvants include traditional mineral salt-based adjuvants, oil-in-water/water-in-oil emulsions and saponins. In addition, pathogen-associated molecular patterns, such as Toll like receptor (TLR) ligands, are well-documented immunomodulators that are increasingly recognized as critical components of many modern vaccines. However, the potential of these adjuvants (either traditional or modern ones) to drive the desired type of adaptive immune response when combined to an immunogenic vaccine candidate is generally unknown or poorly characterized in veterinary medicine. Very frequently, the choice of adjuvants is based on theoretical assumptions of their expected type 1 or type 2 polarizing properties, or on their previous use in vaccine formulations in the veterinary or swine field.

Consequently, in the present study, an in vitro porcine bone marrow-derived dendritic cell (bmDC) culture was used as a model for evaluating immune polarization induced by different adjuvants when administrated with immunogens that have the potential to become vaccine candidates if appropriately formulated. We hypothesize that this in vitro model can reduce the number of animals used in pre-clinical trials by providing fundamental immunological knowledge on selected vaccine formulations (from several possible ones) that would deserve further analysis in animal trials. To develop this in vitro system, different *S. suis* antigens (enolase, CPS and its conjugated form) were used to evaluate whether the in vitro bmDC culture can differentiate the immunogenic potential of the antigen in combination with different polarizing adjuvants.

2. Results

2.1. Characterization of BmDCs Differentiated by in House-Prepared Porcine GM-CSF

Expression of cell surface markers of swine bmDCs was assessed by FACS after eight days of culture with the in house-produced porcine granulocyte-macrophage colony-stimulating factor (pGM-CSF) supernatant in comparison to commercial swine recombinant (r)GM-CSF (Table 1). Cells were shown to be MHC-I+, MHC-II+, SWC3+, CD1+, CD16+, CD14+, CD11R1− and CD4alow/−, as previously described (31, 32). Dose-response studies showed that final 1/50 dilution of pGM-CSF supernatant was optimal for the generation of

bmDCs in vitro (data not shown). No significant differences were observed in bmDC phenotype obtained by culturing in the presence of in house-prepared pGM-CSF compared to that obtained when using commercial rGM-CSF (Table 1). These results suggest that the use of a CHO-K1/pGM-CSF stable cell line to generate the growth factor is an efficient and low-cost alternative to use of commercial rGM-CSF, especially when large numbers of bmDCs are required. Indeed, the total yield of differentiated bmDCs per animal was of approximately 1×10^8 bmDCs.

2.2. Dose Response and Cytotoxicity of Selected Adjuvants

When using adjuvants to activate immune cells in a closed system, as in the case of a culture well, the main challenge is to find the optimal concentration that will preserve the adjuvant properties but with the least possible toxic effect towards cells. To this aim, bmDCs were activated with different concentrations of the following adjuvants: Poly I:C (a synthetic double stranded RNA that activates multiple elements of the host defense, mainly through recognition by TLR3 (33)); Quil A ® (a saponin belonging to the group of glycosides commonly found in plants that have been tested and commercialized for use in animals (29)); and Alhydrogel ® (an aluminium-based adjuvant, generically referred to as “alum”, already described in 1926 and currently the most widely used in humans and animals (34)). In addition, different added volumes of the emulsifying adjuvants TiterMax Gold ® and Stimune ® were evaluated. TiterMax Gold ® is a water-in-oil adjuvant consisting of squalene as metabolizable oil, sorbitan monooleate 80 as an emulsifier, CRL8300 block copolymer and microparticulate silica as stabilizers. TiterMax Gold ® was developed as a superior alternative to Freund’s adjuvant providing comparable titers with fewer injections and less undesired reactivity in mice (35). Stimune ® (also known as Specol) is a water-in-oil adjuvant composed of purified and defined mineral oil (Marcol 52) with Span 85 and Tween 85 as emulsifiers (36).

Cell toxicity and cytokine production were assessed in parallel. The concentration of Poly I:C (50 μg/mL) was chosen according to Mussa et al. (37), and induced very low cell toxicity (Figure S1B). In spite of low to moderate toxicity, the highest production of cytokines was obtained with 5 μg/mL of Quil A ®, 50 μg/mL of Alhydrogel ®, and 100 μL/well of

TiterMax Gold ® or Stimune ® emulsions (Figures S1A,B). Induction of some level of toxicity is an adjuvant feature also observed in vivo (36, 38) that might also contribute to the activation of immune cells at the site of the injection. Regardless of this effect in our in vitro culture system, a clear enhancement and polarization of DC activation is observed when combining the adjuvants with an antigen (see below), suggesting that culture conditions were adequately standardized.

2.3. Adjuvants Intensify the BmDC Activation Potential of a Protein Antigen

To determine the ability of adjuvants to increase the activation of bmDCs induced by the vaccine candidate enolase, each adjuvant alone or in combination with enolase (at the selected doses; Figures S1 and S2) were incubated with bmDCs during 24 h, and then the production of type 1 or type 2 signature cytokines (namely IL-12, TNF-α and IL-6) was measured. As shown in Figure 1, enolase alone induced significant levels of the three evaluated cytokines when compared to non-activated cells (used as negative control). The adjuvant Poly I:C alone failed to induce significant levels of cytokine production by bmDCs. However, when combined with enolase a synergistic effect on TNF-α and, to a lesser extent, on IL-12 production is observed (Figure 1A,B), whereas levels of IL-6 were reduced (Figure 1C). The adjuvant Quil A ® alone induced high levels of IL-12 production by bmDCs, which was not further increased by the presence of enolase. Nevertheless, a significant synergistic effect is observed between enolase and Quil A ® for TNF-α production (Figure 1A,B). Similarly to Poly I:C, enolase-induced IL-6 production is reduced in the presence of Quil A ® (Figure 1C).

The adjuvant Alhydrogel ® alone induced high levels of IL-12 production by bmDCs, which was not further increased by the presence of enolase. On the other hand, this adjuvant induced low levels of TNF-α production and did not modify those induced by enolase (Figure 1A,B). In contrast to Poly I:C and Quil A ® adjuvants, Alhydrogel ® induced high levels of IL-6 production and synergistically enhanced this cytokine release by enolase-activated bmDCs (Figure 1C).

The emulsifying adjuvants TiterMax Gold ® and Stimune ® alone failed to induce IL-12 and TNF-α release by bmDCs. Moreover, they markedly reduced the production of these two cytokines by enolase-activated bmDCs (Figure 1A,B). Nevertheless, and similarly to Alhydrogel ®, emulsifying adjuvants induced high levels of IL-6 production and showed a synergistic effect on this cytokine release by enolase-activated bmDCs (Figure 1C).

These results suggest that adjuvants, in combination with enolase, intensify bmDC activation, although different cytokine patterns are generated.

2.4. BmDCs Can Distinguish Type 1 vs. Type 2 Adjuvants in Combination with Enolase

As different patterns of cytokines were observed depending on the adjuvant used in combination with enolase, a type 1 (IL-12 or TNF-α) vs. type 2 (IL-6) profile was tentatively established and statistically analyzed. Results of these analyses are displayed in Figure 2 by either using IL-12/IL-6 or TNF-α/IL-6 comparative expression. Independently of the cytokine combination used for the analyses, enolase alone induced a mixed type1/type 2 cytokine pattern. Poly I:C and Quil A ® biased this response towards a type 1 phenotype by reducing IL-6 production and favoring IL-12 and TNF-α production by bmDCs. Alhydrogel ® amplified enolase-induced IL-6 production, although TNF-α levels were maintained and a strong induction of IL-12 release was also observed, altogether suggesting a mixed type1/type2 profile. In contrast, the emulsifying adjuvants TiterMax Gold ® and Stimune ® significantly diminished enolase-induced production of IL-12 and/or TNF-α and favoured IL-6 production when compared to enolase alone. These latter observations suggest a type 2 bias of bmDC response to enolase with the two emulsifying adjuvants tested.

As shown by the letters in Figure 2, an inter-adjuvant statistical analysis showed that Quil A ® and Alhydrogel ® have the highest capacity to favour IL-12 production by porcine bmDCs. Quil A ® also has the highest capacity to influence TNF-α production, a shared feature with Poly I:C. On the other hand, Alhydrogel ®, TiterMax Gold α and Stimune ® were equally powerful inducers of IL-6.

2.5. BmDC Cytokine Response Is Differentially Modulated upon the Chemical Nature of Antigens, Which in Turn Partially Affected the Polarizing Activity of Adjuvants

To determine the ability of bmDCs to discriminate the immunogenic potential of antigens with diverse chemical nature (polysaccharide, protein or polysaccharide-protein), the effects of CPS, tetanus toxoid protein (TT) or a CPS-TT conjugate were evaluated. As expected (39) and due to its polysaccharide nature, CPS alone failed to induce significant levels of cytokine release by porcine bmDCs (Figure 3). The protein TT induced high levels of TNF-α and IL-6, but non-significant levels of IL-12. The CPS-TT conjugate slightly increased IL-12 production when compared to non-stimulated control cells. Compared to CPS alone or control cells, the conjugate also induced higher levels of TNF-α and IL-6. However, levels of these two cytokines were lower than those induced by TT alone, suggesting that the CPS modifies TT activity when the two components are conjugated together.

To determine if the capacity of bmDCs to distinguish the polarizing effect of adjuvants depends on the chemical nature of the antigen or the identity of the antigen, type 1 (IL-12 or TNF-α) vs. type 2 (IL-6) profiles were analyzing using bmDCs activated with CPS, TT, or CPS-TT conjugate in combination with different adjuvants. As shown in Figure 4, in contrast to the results using a protein antigen, Poly I:C failed to overcome CPS unresponsiveness and to polarize the immune response of this polysaccharide antigen. In the case of Quil A ®, no clear polarizing effect on CPS capacity to activate bmDCs was observed, and IL-12 and TNF-α production by bmDCs seemed to be mainly related to the intrinsic capacity of this adjuvant to induce the release of these cytokines (Figures 1 and 4). Similarly, the mixed type1/type2 profile induced by Alhydrogel ® and the type 2 cytokine profile induced by the two emulsifying adjuvants were not modified/amplified in the presence of CPS (Figures 1 and 4).

On the other hand, a bias towards a type 1 phenotype was observed when either TT or CPS-TT conjugate were combined with Poly I:C or Quil A ®, as previously observed with enolase, with a clear suppression of IL-6 production by bmDCs. Nevertheless, the capacity of these adjuvants to enhance TNF-α and/or IL-12 production by TT-stimulated bmDCs was limited when compared to the effect observed in combination with enolase. Furthermore, Quil A ®-intrinsic capacity to induce production of IL-12 was partially impaired in the presence of

TT ($p < 0.05$), but not in the presence of the CPS-TT conjugate (Figure 4). A similar effect on Alhydrogel ®-induced IL-12 production was observed when combined with TT, although a mixed type1/type2 response was still obtained when using Alhydrogel ® + TT or Alhydrogel ® + CPS-TT conjugate formulations (Figure 4). Finally, in spite of a clear suppressive effect on TNF-α production, the emulsifying adjuvants TiterMax Gold ® and Stimune ® failed to provide a type 2 (IL-6) adjuvating effect when formulated with TT, compared to that induced by TT alone. On the other hand, these adjuvants when emulsified with the CPS-TT conjugate promote a bias to a type 2 response, with moderate, but significant, increase in IL-6 production (Figure 4). These results suggest that the amplitude of the adjuvant effect might depend on the chemical nature of the antigen.

3. Discussion

The present work developed a model of porcine bmDC in vitro culture for analyzing sub-unit vaccine candidates with previously reported immunogenic potential in combination with various adjuvants for the control of *S. suis* infections. The use of this model provided fundamental knowledge on the polarizing effect of the adjuvant and thus the expected benefits if included in a vaccine formulation. The generated information would potentially contribute to reducing the number of animals used in pre-clinical trials. Furthermore, this study provides for the first time specific information on swine DC immune activation by the adjuvants Quil A ®, Alhydrogel ®, TiterMax Gold ® and Stimune ®. This information is highly significant as species-specific responses might be obtained for a given adjuvant, as described below.

In our porcine bmDC in vitro culture system, Poly I:C and Quil A ® behave as type 1 polarizing adjuvants. Interestingly, Poly I:C lacks intrinsic capacity to induce the release of cytokines (at least those evaluated in this study), but it is able to polarize the cytokine profile induced by the antigen. Similarly, it has been reported that this TLR-ligand possesses weak or no stimulatory ability by itself (37, 40), but enhances the release of IL-12 by porcine bmDCs stimulated by the bacterial pathogen *Haemophilus parasuis* (37). In contrast to swine DCs, human monocyte-derived DCs, purified human CD11c+ myeloid DCs, or murine bmDCs produce significant levels of IL-12p40/p70 after Poly I:C stimulation (41-43). In addition,

mouse origin DCs also respond with significant levels of TNF-α or IL-6 release after stimulation with this TLR ligand (43-45). In contrast to Poly I:C, Quil A® alone induces the release of high levels of IL-12 by swine bmDC and, consequently, biases cell activation towards a type 1 profile when combined with an antigen in vitro. Nevertheless, it is unclear whether inter-species differences are also expected in Quil A ® capacity to modulate DC activation in vitro as no studies are available with human or mouse origin DCs.

Several vaccination trials in swine, including one against *S. suis*, reported the use of Quil A ® as adjuvant (22, 46, 47). In a field swine immunization study, Quil A ® induced a biased response towards IgG2 directed against *Taenia solium* antigens (46). Swine immunization with recombinant *S. suis* Sao protein formulated with Quil A ® conferred protection against *S. suis* infection, which was correlated with a predominant IgG2 response (22). Thus, studies suggest that Quil A ® behaves as a type 1 adjuvant in swine, and our in vitro data supports this concept. Albeit not completely characterized, when used as adjuvant in swine anti-viral vaccines (48-50), Poly I:C was suggested to induce a type 1 protective response; results with porcine bmDCs confirm this hypothesis, at least when combined with protein antigens.

In contrast to swine immunization models, either a type 1 or a mixed type1/type 2 response is generally reported in mouse models when immunizing with Quil A ® or Poly I:C in combination with several antigens (22, 51-53), supporting the aforementioned inter-species differences observed in vitro. In this regard, *S. suis* enolase formulated with Quil A ® triggered a balanced type 1/type2 profile in mice (12). As immunization with enolase + Quil A ® failed to protect mice against a *S. suis* challenge, it would be interesting to evaluate if this formulation has a better protective effect in swine, whereas a more marked bias to a type 1 response would be expected.

The cellular and molecular pathways involved in ‘alum’ mechanism of action have been extensively studied (34, 54). A significant consideration in the selection of alum as an adjuvant is that alum is universally considered to preferentially support a Th2 immune response, mainly based on mouse models (34, 54, 55). Immunization of mice with different *S.*

suis immunogenic proteins with alum also suggests a favoured induction of a type 2 antibody response (10). Fewer studies have analyzed alum-polarizing effects in swine. Although an IgG2 response is also observed in pigs vaccinated with alum-adjuvanted vaccines, a higher IgG1/IgG2 ratio is observed, suggesting a predominant Th2 immune response (56, 57). In vitro, swine DCs responded to alum with a mixed type1/type2 cytokine profile. It has been proposed that alum mainly stimulates a Th2 immune response without affecting the Th1 response (57). Using different immunization protocols, a study showed that type 1 and type 2 components were present in all protocols, and it was the balance between the opposing cytokines that determined the final outcome of the humoral response in vivo (58). Thus, the alum polarizing effect remains to be fully characterized in swine models, and, more particularly, for *S. suis* antigens.

Using the in vitro culture of porcine bmDCs, we showed that the water-in-oil adjuvants TiterMax Gold ® and Stimune ® polarize the immune response towards a type 2 phenotype. Stimune ® has been largely used in veterinary medicine, including swine vaccination trials against S. suis (10, 15, 59, 60). However, the polarizing effect of this adjuvant after swine immunization has been poorly addressed (10). A recently reported pig vaccination trial with the S. suis CPS-TT conjugate prototype adjuvanted in Stimune ® showed a preferential IgG1 isotype switch (15), confirming our in vitro observations with swine DCs. Studies in mice also showed that Stimune ® preferentially polarizes the humoral response towards the IgG1 class when combined with either a peptide antigen (61) or a glycoconjugate vaccine (62). As indicated above, Stimune ® is a Marcol 52-based emulsion, and, when this mineral oil is used as adjuvant in combination with different *S. suis* immunogenic proteins, it has also been shown to induce a Th2-biased antibody response in mice (10). In contrast, TiterMax Gold ® is a squalene-based adjuvant which has been reported to induce either mixed Th1/Th2 or Th2-polarized humoral responses in mice (15, 63, 64). Squalene based oil-in-water emulsions (MF59 and AS03) licensed for human use also stimulate a mixed Th1/Th2 cell phenotype (26). Data on the adjuvant effect of squalene-based emulsions in swine (in vitro or in vivo) are scarce; however, a study suggested that an AS03-based vaccine might induce a Th2 response in swine (65), supporting our hypothesis that TiterMax Gold ® polarize swine DC responses

towards a type 2 phenotype. Further studies in swine with these types of emulsions will provide more information on their adjuvant effect.

In a vaccine formulation, not only the adjuvant but also the antigen has the potential to modulate DC functions and the overall adaptive immune response generated by the vaccine. In this study, the polarizing effect of the adjuvants was similar when combined with two different proteins or with a glycoconjugate. However, the magnitude of the response varies, highlighting the need to carefully evaluate the intrinsic properties of the antigen when choosing the adjuvant to be incorporated in the vaccine formulation. Similarly to our in vitro results with DCs, mouse immunization studies suggested that the effects of the adjuvants might be antigen-dependent (54, 64). Finally, we demonstrated in vitro that different adjuvants were unable to overcome the poor immunogenicity of purified *S. suis* serotype 2 CPS, a finding that was confirmed in mouse immunization studies (15).

Our data also showed that the overall DC activation might be the result of a simple additive effect or a synergistic effect between the antigen and the adjuvant, or an effect induced by the adjuvant component alone. In addition, when a bias is imposed by the adjuvant, there may be a suppressive effect over a cytokine with consequent enhancement of another one. Within a vaccine formulation, the combination of all these features will dictate the final capacity of the vaccine formulation to modulate DC functions. Signaling pathways induced by adjuvants and involved in the different responses observed are complex and out of the scope of our work; nevertheless, our data and those reported in the literature highlight the risk in directly transferring the results observed in other systems (either mouse or human) to swine systems.

The present work suggests that swine bmDCs are able to discriminate the polarizing effect of adjuvants in combination with different antigens, according to the cytokine profile observed. This in vitro model was also able to distinguish (with respect to the profile and/or magnitude of the cytokine response) the intrinsic activating capacity of antigens with diverse chemical natures. Although results still remain to be validated in vivo, using swine immunizations, this model represents a valuable tool to examine the immunogenic potential of

vaccine candidates while also screening for adjuvants favouring the desired immune response. In addition to the importance of the basic immunological knowledge generated by this in vitro test, by pre-screening formulations for likelihood of success in vivo, this model has the potential to promote the "Three Rs" ("Replacement, Reduction and Refinements") guiding principle for more ethical use of animals. As recently stated by Perrie et al. (66), an adjuvant or vaccine formulation that fails to stimulate DC activation in vitro is not likely to be successful in vivo. Dissecting the interaction of antigens and adjuvants in vitro with professional APCs (such as DCs) is a simple but promising approach that helps to assess the behaviour of a vaccine formulation rapidly. Ultimately, animal testing of leading formulation(s) will be essential (66).

4. Materials and Methods

4.1. Purification of S. suis Enolase

Cloning and purification of enolase were performed as previously described (11). Briefly, the gene coding for enolase was amplified by PCR and was cloned into pET-32a vector (Novagen, Madison, WI, USA). The primers used were the forward primer 5′-TATAA GGATCC TTGTCAATTATTACTGATGTTTACGC-3′, introducing a BamHI site (underlined letters), and the reverse primer 5′-TATA AAGCTT TTATTTTTTCAAGTTGTAGAATGAGTTCAAGCC-3′, introducing a HindIII site (underlined letters). PCR reactions were carried out with Phusion ® high fidelity taq polymerase (New England Biolabs, Ipswich, MA, USA). PCR amplification conditions consisted of 2 min at 98 °C and 35 cycles of 10 s at 98 °C, 10 s at 56 °C, and 60 s at 72 °C using a Biometra TGradient thermocycler (Göttingen, Germany) and a final elongation step of 5 min. Plasmid was purified by mini-prep (Qiagen, Toronto, ON, Canada). Purified plasmid was digested with BamHI and HindIII in order to confirm presence of insert. Automated sequencing was used to check the amplified enolase gene. The verified complete gene was cloned into pET-32a vector using the BamHI and HindIII sites. This plasmid contains a His-tag-encoding sequence of about 25 kDa. The plasmid pET-32a-Enolase was introduced into E. coli Bl21DE3 for IPTG-inducible expression of recombinant enolase. The protein was purified by His-Bind ® Resin chromatography (EMD Millipore Corp, Billerica, MA, USA) through

the His-tagged fusion according to the manufacturer's protocol. Protein-containing fractions were determined by NanoDrop ® ND-1000 (Thermo-Fisher Scientific, Waltham, MA, USA). The purity of enolase fractions was verified by Western blotting using optimally diluted monospecific rabbit anti-enolase IgG and peroxidase-conjugated goat anti-rabbit (Jackson ImmunoResearch, Baltimore, PA, USA), as previously described (11). Final protein concentration was measured by PierceTM BCA Protein Assay kit (Thermo-Fisher Scientific).

4.2. Production and Purification of S. suis CPS and Its Conjugate

S. suis serotype 2 CPS was produced and purified as previously described (39, 67). Preparation of CPS and TT, covalent coupling of CPS to TT and characterization of the obtained glycoconjugate was performed as previously described (15). Briefly, purified CPS was depolymerized by ultrasonic irradiation to a MW of 115 kDa, and then 10% of the capsular sialic acids (Neu5Ac) were mildly oxidized by treating with sodium periodate in order to introduce functional aldehydes for subsequent conjugation to TT, used as the carrier protein. The TT monomer was purified by gel filtration chromatography before conjugation. The glycoconjugate was produced using a molar mixture of 2 oxidized CPS chains:1 TT, which was conjugated by reductive amination for 48 h. The conjugation was stopped by the addition of sodium borohydride to reduce the remaining free aldehydes for 1 h. The resulting conjugate was desalted by extensive dialysis against water and lyophilized. To obtain a representative control of unconjugated CPS for this study, a sample of the same oxidized CPS used for conjugation was reduced, desalted and lyophilized in the same manner as the conjugate. Purified TT was also included as control.

4.3. Production of in House pGM-CSF

CHO-K1/pGM-CSF stable cell line was derived from the CHO-K1 cell line (ATCC ® CCL-61TM, Manassas, VA, USA). A 459 bp DNA fragment corresponding to the coding region of Sus scrofa colony stimulating factor 2 (CSF2/GM-CSF) mRNA (accession number NM_214118) was chemically synthesized (Genescript, Piscataway, NJ, USA). The synthetized DNA fragment was then cloned into the mammalian expression vector pcDNA3.1(+) using EcoRI (upstream) and XhoI (downstream) as enzymes to make the expression construct. The final expression construct was sequenced (sequencing primer

TGGGAGGTCTATATAAGCAGAG) and was 100% accurate with CSF2/GM-CSF template sequence. CHO-K1 cells were then transfected with this final construct encoding pGM-CSF and stable clones were obtained by selection using geneticin at 600 µg/mL (G418, Gibco, Invitrogen, Thermo-Fisher Scientific). A number of positive clones were selected for further evaluation after culturing for 10 passages. Based on Western blot analysis with monoclonal anti-porcine GM-CSF antibody (MAB711; R&D systems, Mineapolis, MN, USA), clones 10, 25 and 30 were selected for final use. For pGM-CSF production, CHO-K1/pGM-CSF cells were suspended at 5 × 105 cellules/mL in DMEM F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-Glutamine and 600 µg/mL of geneticin (600 µg/mL). All reagents were from Gibco. Cells were initially cultured in T25 flasks and then sub-cultured in T75 flasks (Sarstedt, Nümbrecht, Germany) at 37 °C in a 5% CO2 incubator for approximately two days until confluence. For sub-culturing, cells were washed with a 2% of EDTA-PBS solution and detached with 0.25% of trypsin for 1 min. For final pGM-CSF production, cells were cultured in T75 flasks in complete medium without geneticin for five days. Finally, supernatants were recovered and centrifuged twice at 830 g for 10 min at room temperature. The supernatant was recovered, aliquoted and stored at −80 °C.

4.4. Animals and Isolation of Porcine Bone Marrow Cells

Bone marrow cells were obtained from 6–8 week-old piglets (n = 10). The animals originated from a herd free of major important diseases such as porcine reproductive and respiratory syndrome, enzootic pneumonia due to Mycoplasma hyopneumoniae and clinical disease related to porcine circovirus. The herd did not have any episode of acute disease related to S. suis when the samples were taken. All experiments involving animals were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals and approved by the Animal Welfare Committee of the University of Montreal (protocol # 2016-Rech-1570). Bone marrow was aseptically removed from the femurs of ten different animals and separately processed using endotoxin-free solutions and materials. Briefly, after removal of muscle tissue, femurs were sliced and stirred in 1 L of PBS for 2 h at room temperature.

The PBS suspension containing released bone marrow cells was recovered, filtered through gauzes and centrifuged at 250 g for 10 min at 4 °C. After red blood cell lysis (eBioScience, San Diego, CA, USA), cells were washed, filtered through a 40 μm-cell strainer (BD FalconTM, Bedford, MA, USA), and resuspended at approximately 1–3 × 107 cells/mL in a cryopreservation solution containing 95% of FBS and 5% of dimethylsulfoxyde (DMSO) (Sigma-Aldrich, St Louis, MO, USA) and stored in liquid nitrogen until use.

4.5. Generation of Porcine BmDCs

Bone marrow cells were thawed and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U/mL penicillin-streptomycin, and 1 □g/mL gentamycin. All reagents were from Gibco. Complete medium was complemented with either 1/50 dilution of pGM-CSF supernatant or with 100 ng/mL commercial porcine rGM-CSF (R&D system), as previously described (32). Then, 2.5 × 106 cells/well were cultured in 6 well-tissue culture plates (Falcon ®, Corning, Tewksbury, MA, USA) for 8 days at 37 °C in a 5% CO2 incubator and were fed on days 3 and 6 with fresh complete medium. On day 8, cells were harvested, washed, and used as immature DCs for the studies. DC phenotype and purity was confirmed by FACS as described below.

4.6. DC Phenotype Analysis by FACS

Obtained porcine bmDCs were phenotypically characterized by the following markers: MHC-I, MHC-II, CD4a, CD16, CD14, CD11R1, SWC3 and CD1, as previously described (32, 68). Commercially available monoclonal antibodies from AbD Serotec (Kidlington, Oxford, United Kingdom) were used to detect swine MHC-I (clone JM1E3), MHC-II (clone 2E9/13), CD16 (clone G7), and CD4a (MIL17). Monoclonal antibody against swine CD11R1 (clone MIL4) and CD14 (clone MIL2) were from Bio-Rad (Kidlington, Oxford, United Kingdom). Monoclonal antibody against swine CD1 (clone 76-7-4) was from Abcam Inc (Cambridge, MA, USA). A hybridoma specific for SCW3 was used (clone 74.22-15A; ATCC HB-142.1). Antibodies against MHC-I, MHC-II, CD16, CD1 and CD4a were conjugated to FITC. A PE-conjugated goat anti-mouse IgG (Leinco Technologies Inc, St Louis, MO, USA) was used for labelling CD11R1, CD14 and SCW3. The staining and FACS analysis were

performed as previously described for swine bmDCs (32) using a FACS BD Accuri C6 Cytometer (BD Biosciences, San Jose, CA, USA).

4.7. In Vitro BmDC Stimulation Assay

Bone marrow DCs were resuspended at 106 cells/ml in complete medium and plated into 24 well-culture plate (Falcon ®). Then, different activators were added. Four different antigens were used: enolase at 50 μg/mL (the concentration was chosen based on a preliminary dose response study, see Figure S2); CPS at 25 μg/mL; TT at 25 μg/mL; CPS-TT conjugate at 25 μg/mL (these concentrations were chosen based on (15)). Several categories of adjuvants were evaluated: the TLR-ligand Poly I:C (Novusbio, Oakville, ON, Canada), was added at 50 μg/mL; the saponin Quil A ® (Brenntag Biosector, Frederikssund, Denmark), was added at 5 μg/mL; Alhydrogel 2% ® (Brenntag Biosector), composed of aluminum hydroxide, was added at 50 μg/mL; the water-in-oil emulsion TiterMax Gold ® (Cytrx Corporation, Norcross, GA, USA) emulsified 1:1 (v/v) with antigens and the water-in-oil emulsion Stimune ® (Prionics, Lelystad, The Netherlands) emulsified 5:4 (v/v) with antigens were added at 100 □L/well to culture plates. Concentrations of different adjuvants were chosen upon a preliminary selection based on the literature (12, 15, 22, 37, 61, 69-72) and finally established by a dose response study of toxicity levels and cytokine production (Figure S1). The preparation of adjuvant–antigen mixtures followed the manufacturer's recommendations. To avoid any influence of possible endotoxin contamination during cell stimulation assays, polymyxin B sulfate (Sigma) at 20 μg/mL was added to the cultures. At 24 h of incubation, supernatants were collected for cytokine quantification by ELISA, as described below.

4.8. Cytokine Quantification by ELISA and Cell Toxicity Test

Levels of IL-12p40, TNF-α and IL-6 in cell culture supernatants were measured by sandwich ELISA using pair-matched antibodies from R&D Systems or Invitrogen according to the manufacturer's recommendations. Twofold dilutions of recombinant porcine cytokines were used to generate the standard curves. Sample dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine. Cell toxicity induced by different adjuvants was evaluated by measuring release of

lactate dehydrogenase enzyme (LDH) with the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) as previously described (73).

4.9. Statistical Analysis

Data were transformed using the logarithm base 10 to normalize distributions. A linear mixed model was used to compare the various treatments. The random factor was the animal and the fixed factor was treatment. The model also included uneven variances for the various treatments. A priori contrasts were performed to compare pairs of means adjusting the alpha level for each comparison using the Benjamini–Hochberg sequential adjustment procedure. The nominal alpha level was set at 0.05 throughout. Statistical analyses were carried out with SAS v.9.4 (Cary, NC, USA). This method was applied for analysis of data displayed in Figures 1 and 2 (n = 10). All other data were analyzed for significance using a Student's unpaired t-test, SigmaPlot (version 11.0, Systat Software, San Jose, CA). A p value < 0.05 was used as a threshold for significance.

5. Conclusions

In conclusion, use of in vitro methods as a replacement for animal models at the pre-screening stages will certainly reduce numbers of animals used while advancing the vaccinology science. This approach can be used to test other *S. suis* vaccine candidates and accelerate the design of promising sub-unit vaccines against this pathogen.

Acknowledgments

This publication was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant to MS (# 342150). Partial support was also obtained from the Swine and Poultry Infectious Diseases Research Centre ('CRIPA'). L.M. and G.G-D. are recipients of fellowships from CRIPA; G.G-D. is also the recipient of an NSERC PhD Award.

References

1. Gottschalk M, Xu J, Calzas C, Segura M. Streptococcus suis: a new emerging or an old neglected zoonotic pathogen? Future Microbiol. 2010;5(3):371-91.

2. Segura M. Streptococcus suis: an emerging human threat. J Infect Dis. 2009;199(1):4-6.

3. Okura M, Osaki M, Nomoto R, Arai S, Osawa R, Sekizaki T, et al. Current taxonomical situation of Streptococcus suis. Pathogens. 2016;5(3).

4. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. Streptococcus suis, an important pig pathogen and emerging zoonotic agent—an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect. 2014;3:e45.

5. Gottschalk M. Streptococcosis. In: Zimmerman JJ, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of swine. 10th ed. Ames, USA: Wiley-Blackwell Publishing; 2012. p. 841-55.

6. Baums CG, Bruggemann C, Kock C, Beineke A, Waldmann KH, Valentin-Weigand P. Immunogenicity of an autogenous Streptococcus suis bacterin in preparturient sows and their piglets in relation to protection after weaning. Clin Vaccine Immunol. 2010;17(10):1589-97.

7. Segura M, Calzas C, Grenier D, Gottschalk M. Initial steps of the pathogenesis of the infection caused by Streptococcus suis: fighting against non-specific defenses. FEBS Lett. 2016.

8. Fittipaldi N, Segura M, Grenier D, Gottschalk M. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent Streptococcus suis. Future Microbiol. 2012;7(2):259-79.

9. Gottschalk M, Segura M. The pathogenesis of the meningitis caused by Streptococcus suis: the unresolved questions. Vet Microbiol. 2000;76(3):259-72.

10. Segura M. Streptococcus suis vaccines: candidate antigens and progress. Expert Rev Vaccines. 2015;14(12):1587-608.

11. Esgleas M, Li Y, Hancock MA, Harel J, Dubreuil JD, Gottschalk M. Isolation and characterization of alpha-enolase, a novel fibronectin-binding protein from Streptococcus suis. Microbiology. 2008;154(Pt 9):2668-79.

12. Esgleas M, Dominguez-Punaro MC, Li Y, Harel J, Dubreuil JD, Gottschalk M. Immunization with SsEno fails to protect mice against challenge with Streptococcus suis serotype 2. FEMS Microbiol Lett. 2009;294(1):82-8.

13. Zhang A, Chen B, Mu X, Li R, Zheng P, Zhao Y, et al. Identification and characterization of a novel protective antigen, Enolase of Streptococcus suis serotype 2. Vaccine. 2009;27(9):1348-53.

14. Feng Y, Pan X, Sun W, Wang C, Zhang H, Li X, et al. Streptococcus suis enolase functions as a protective antigen displayed on the bacterial cell surface. J Infect Dis. 2009;200(10):1583-92.

15. Goyette-Desjardins G, Calzas C, Shiao TC, Neubauer A, Kempker J, Roy R, et al. Protection against Streptococcus suis serotype 2 infection using a capsular polysaccharide glycoconjugate vaccine. Infect Immun. 2016;84(7):2059-75.

16. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol. 1995;13:251-76.

17. Shibuya K, Robinson D, Zonin F, Hartley SB, Macatonia SE, Somoza C, et al. IL-1 alpha and TNF-alpha are required for IL-12-induced development of Th1 cells producing high levels of IFN-gamma in BALB/c but not C57BL/6 mice. Journal of immunology (Baltimore, Md : 1950). 1998;160(4):1708-16.

18. Mihara M, Hashizume M, Yoshida H, Suzuki M, Shiina M. IL-6/IL-6 receptor system and its role in physiological and pathological conditions. Clin Sci (Lond). 2012;122(4):143-59.

19. Diehl S, Rincon M. The two faces of IL-6 on Th1/Th2 differentiation. Mol Immunol. 2002;39(9):531-6.

20. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. Front Immunol. 2014;5:520.

21. Guilliams M, Bruhns P, Saeys Y, Hammad H, Lambrecht BN. The function of Fc gamma receptors in dendritic cells and macrophages. Nat Rev Immunol. 2014;14(2):94-108.

22. Li Y, Gottschalk M, Esgleas M, Lacouture S, Dubreuil JD, Willson P, et al. Immunization with recombinant Sao protein confers protection against Streptococcus suis infection. Clin Vaccine Immunol. 2007;14(8):937-43.

23. Michaelsen TE, Kolberg J, Aase A, Herstad TK, Hoiby EA. The four mouse IgG isotypes differ extensively in bactericidal and opsonophagocytic activity when reacting with the P1.16 epitope on the outer membrane PorA protein of Neisseria meningitidis. Scand J Immunol. 2004;59(1):34-9.

24. Crawley A, Raymond C, Wilkie BN. Control of immunoglobulin isotype production by porcine B-cells cultured with cytokines. Vet Immunol Immunopathol. 2003;91(2):141-54.

25. Crawley A, Wilkie BN. Porcine Ig isotypes: function and molecular characteristics. Vaccine. 2003;21(21–22):2911-22.

26. Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity. 2010;33(4):492-503.

27. Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunol Cell Biol. 2004;82(5):488-96.

28. Li Y, Martinez G, Gottschalk M, Lacouture S, Willson P, Dubreuil JD, et al. Identification of a surface protein of Streptococcus suis and evaluation of its immunogenic and protective capacity in pigs. Infect Immun. 2006;74(1):305-12.

29. Gerdts V. Adjuvants for veterinary vaccines--types and modes of action. Berl Munch Tierarztl Wochenschr. 2015;128(11-12):456-63.

30. Heegaard PM, Fang Y, Jungersen G. Novel adjuvants and immunomodulators for veterinary vaccines. Methods in molecular biology. 2016;1349:63-82.

31. Carrasco CP, Rigden RC, Schaffner R, Gerber H, Neuhaus V, Inumaru S, et al. Porcine dendritic cells generated in vitro: morphological, phenotypic and functional properties. Immunology. 2001;104(2):175-84.

32. Lecours MP, Segura M, Lachance C, Mussa T, Surprenant C, Montoya M, et al. Characterization of porcine dendritic cell response to Streptococcus suis. Vet Res. 2011;42:72.

33. Martins KA, Bavari S, Salazar AM. Vaccine adjuvant uses of poly-IC and derivatives. Expert Rev Vaccines. 2015;14(3):447-59.

34. Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol. 2009;9(4):287-93.

35. Bennett B, Check IJ, Olsen MR, Hunter RL. A comparison of commercially available adjuvants for use in research. J Immunol Methods. 1992;153(1-2):31-40.

36. Stills HF, Jr. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR J. 2005;46(3):280-93.

37. Mussa T, Rodriguez-Carino C, Sanchez-Chardi A, Baratelli M, Costa-Hurtado M, Fraile L, et al. Differential interactions of virulent and non-virulent H. parasuis strains with naive or swine influenza virus pre-infected dendritic cells. Vet Res. 2012;43:80.

38. Heegaard PM, Dedieu L, Johnson N, Le Potier MF, Mockey M, Mutinelli F, et al. Adjuvants and delivery systems in veterinary vaccinology: current state and future developments. Arch Virol. 2011;156(2):183-202.

39. Calzas C, Goyette-Desjardins G, Lemire P, Gagnon F, Lachance C, Van Calsteren MR, et al. Group B Streptococcus and Streptococcus suis capsular polysaccharides induce chemokine production by dendritic cells via Toll-like receptor 2- and MyD88-dependent and -independent pathways. Infect Immun. 2013;81(9):3106-18.

40. Peng YT, Chaung HC, Chang HL, Chang HC, Chung WB. Modulations of phenotype and cytokine expression of porcine bone marrow-derived dendritic cells by porcine reproductive and respiratory syndrome virus. Vet Microbiol. 2009;136(3-4):359-65.

41. Verdijk RM, Mutis T, Esendam B, Kamp J, Melief CJ, Brand A, et al. Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. Journal of immunology (Baltimore, Md : 1950). 1999;163(1):57-61.

42. Lore K, Betts MR, Brenchley JM, Kuruppu J, Khojasteh S, Perfetto S, et al. Toll-like receptor ligands modulate dendritic cells to augment cytomegalovirus- and HIV-1-specific T cell responses. Journal of immunology (Baltimore, Md : 1950). 2003;171(8):4320-8.

43. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001;413(6857):732-8.

44. Lundberg AM, Drexler SK, Monaco C, Williams LM, Sacre SM, Feldmann M, et al. Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems. Blood. 2007;110(9):3245-52.

45. Jeon YT, Na H, Ryu H, Chung Y. Modulation of dendritic cell activation and subsequent Th1 cell polarization by lidocaine. PloS one. 2015;10(10):e0139845.

46. Jayashi CM, Gonzalez AE, Castillo Neyra R, Kyngdon CT, Gauci CG, Lightowlers MW. Characterisation of antibody responses in pigs induced by recombinant oncosphere antigens from Taenia solium. Vaccine. 2012;30(52):7475-80.

47. Willson PJ, Rossi-Campos A, Potter AA. Tissue reaction and immunity in swine immunized with Actinobacillus pleuropneumoniae vaccines. Can J Vet Res. 1995;59(4):299-305.

48. Thomas M, Wang Z, Sreenivasan CC, Hause BM, Gourapura JR, Li F, et al. Poly I:C adjuvanted inactivated swine influenza vaccine induces heterologous protective immunity in pigs. Vaccine. 2015;33(4):542-8.

49. Zhang L, Bai J, Liu J, Wang X, Li Y, Jiang P. Toll-like receptor ligands enhance the protective effects of vaccination against porcine reproductive and respiratory syndrome virus in swine. Vet Microbiol. 2013;164(3-4):253-60.

50. Cao Y, Lu Z, Li Y, Sun P, Li D, Li P, et al. Poly(I:C) combined with multi-epitope protein vaccine completely protects against virulent foot-and-mouth disease virus challenge in pigs. Antiviral Res. 2013;97(2):145-53.

51. Clausse M, Diaz AG, Ibanez AE, Cassataro J, Giambartolomei GH, Estein SM. Evaluation of the efficacy of outer membrane protein 31 vaccine formulations for protection against Brucella canis in BALB/c mice. Clin Vaccine Immunol. 2014;21(12):1689-94.

52. Xiao C, Rajput ZI, Hu S. Improvement of a commercial foot-and-mouth disease vaccine by supplement of Quil A. Vaccine. 2007;25(25):4795-800.

53. Jansen C, Kuipers B, van der Biezen J, de Cock H, van der Ley P, Tommassen J. Immunogenicity of in vitro folded outer membrane protein PorA of Neisseria meningitidis. FEMS Immunol Med Microbiol. 2000;27(3):227-33.

54. Ghimire TR. The mechanisms of action of vaccines containing aluminum adjuvants: an in vitro vs in vivo paradigm. SpringerPlus. 2015;4:181.

55. Oleszycka E, Lavelle EC. Immunomodulatory properties of the vaccine adjuvant alum. Curr Opin Immunol. 2014;28:1-5.

56. Rivera E, Daggfeldt A, Hu S. Ginseng extract in aluminium hydroxide adjuvanted vaccines improves the antibody response of pigs to porcine parvovirus and Erysipelothrix rhusiopathiae. Vet Immunol Immunopathol. 2003;91(1):19-27.

57. Linghua Z, Xingshan T, Fengzhen Z. The efficacy of CpG oligodinucleotides, in combination with conventional adjuvants, as immunological adjuvants to swine streptococcic septicemia vaccine in piglets in vivo. Int Immunopharmacol. 2006;6(8):1267-76.

58. Comoy EE, Capron A, Thyphronitis G. In vivo induction of type 1 and 2 immune responses against protein antigens. Int Immunol. 1997;9(4):523-31.

59. Wisselink HJ, Vecht U, Stockhofe-Zurwieden N, Smith HE. Protection of pigs against challenge with virulent Streptococcus suis serotype 2 strains by a muramidase-released protein and extracellular factor vaccine. Vet Rec. 2001;148(15):473-7.

60. Wisselink HJ, Stockhofe-Zurwieden N, Hilgers LA, Smith HE. Assessment of protective efficacy of live and killed vaccines based on a non-encapsulated mutant of Streptococcus suis serotype 2. Vet Microbiol. 2002;84(1-2):155-68.

61. Leenaars M, Koedam MA, Hendriksen CF, Claassen E. Immune responses and side effects of five different oil-based adjuvants in mice. Vet Immunol Immunopathol. 1998;61(2-4):291-304.

62. Campodonico VL, Llosa NJ, Bentancor LV, Maira-Litran T, Pier GB. Efficacy of a conjugate vaccine containing polymannuronic acid and flagellin against experimental Pseudomonas aeruginosa lung infection in mice. Infect Immun. 2011;79(8):3455-64.

63. Weeratna RD, McCluskie MJ, Xu Y, Davis HL. CpG DNA induces stronger immune responses with less toxicity than other adjuvants. Vaccine. 2000;18(17):1755-62.

64. Berhanu A, Wilson RL, Kirkwood-Watts DL, King DS, Warren TK, Lund SA, et al. Vaccination of BALB/c mice with Escherichia coli-expressed vaccinia virus proteins A27L, B5R, and D8L protects mice from lethal vaccinia virus challenge. J Virol. 2008;82(7):3517-29.

65. De Vleeschauwer AR, Baras B, Kyriakis CS, Jacob V, Planty C, Giannini SL, et al. Efficacy of an AS03A-adjuvanted split H5N1 influenza vaccine against an antigenically distinct low pathogenic H5N1 virus in pigs. Vaccine. 2012;30(37):5557-63.

66. Perrie Y, Crofts F, Devitt A, Griffiths HR, Kastner E, Nadella V. Designing liposomal adjuvants for the next generation of vaccines. Adv Drug Deliv Rev. 2016;99(Pt A):85-96.

67. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M. Structure determination of Streptococcus suis serotype 2 capsular polysaccharide. Biochem Cell Biol. 2010;88(3):513-25.

68. Summerfield A, McCullough KC. The porcine dendritic cell family. Dev Comp Immunol. 2009;33(3):299-309.

69. Morefield GL, Sokolovska A, Jiang D, HogenEsch H, Robinson JP, Hem SL. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro. Vaccine. 2005;23(13):1588-95.

70. Ghimire TR, Benson RA, Garside P, Brewer JM. Alum increases antigen uptake, reduces antigen degradation and sustains antigen presentation by DCs in vitro. Immunol Lett. 2012;147(1-2):55-62.

71. Cribbs DH, Ghochikyan A, Vasilevko V, Tran M, Petrushina I, Sadzikava N, et al. Adjuvant-dependent modulation of Th1 and Th2 responses to immunization with beta-amyloid. Int Immunol. 2003;15(4):505-14.

72. Leenaars PP, Koedam MA, Wester PW, Baumans V, Claassen E, Hendriksen CF. Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. Lab Anim. 1998;32(4):387-406.

73. Lecours MP, Gottschalk M, Houde M, Lemire P, Fittipaldi N, Segura M. Critical role for Streptococcus suis cell wall modifications and suilysin in resistance to complement-dependent killing by dendritic cells. J Infect Dis. 2011;204(6):919-29.

Tables

Table 1. Surface phenotype of bmDCs after eight days of differentiation using either in house-produced porcine (p)GM-CSF supernatant or commercial recombinant (r)GM-CSF.

Marker	pGM-CSF Supernatant (% Positive Cells) [1]	rGM-CSF Commercial (% Positive Cells) [1]
MHC-I	89 ± 6	90 ± 7
MHC-II	85 ± 6	86 ± 9
SCW3	88 ± 4	86 ± 5
CD1	69 ± 3	67 ± 5
CD16	89 ± 2	93 ± 2
CD14	79 ± 2	84 ± 3
CD4a	14± 2	9 ± 5
CD11R1	4 ± 1	2 ± 1

[1] Mean ± SEM of four independent experiments using the supernatants of three CHO-K1/pGM-CSF stable cell line clones or from three independent experiments using commercial swine rGM-CSF.

Figures

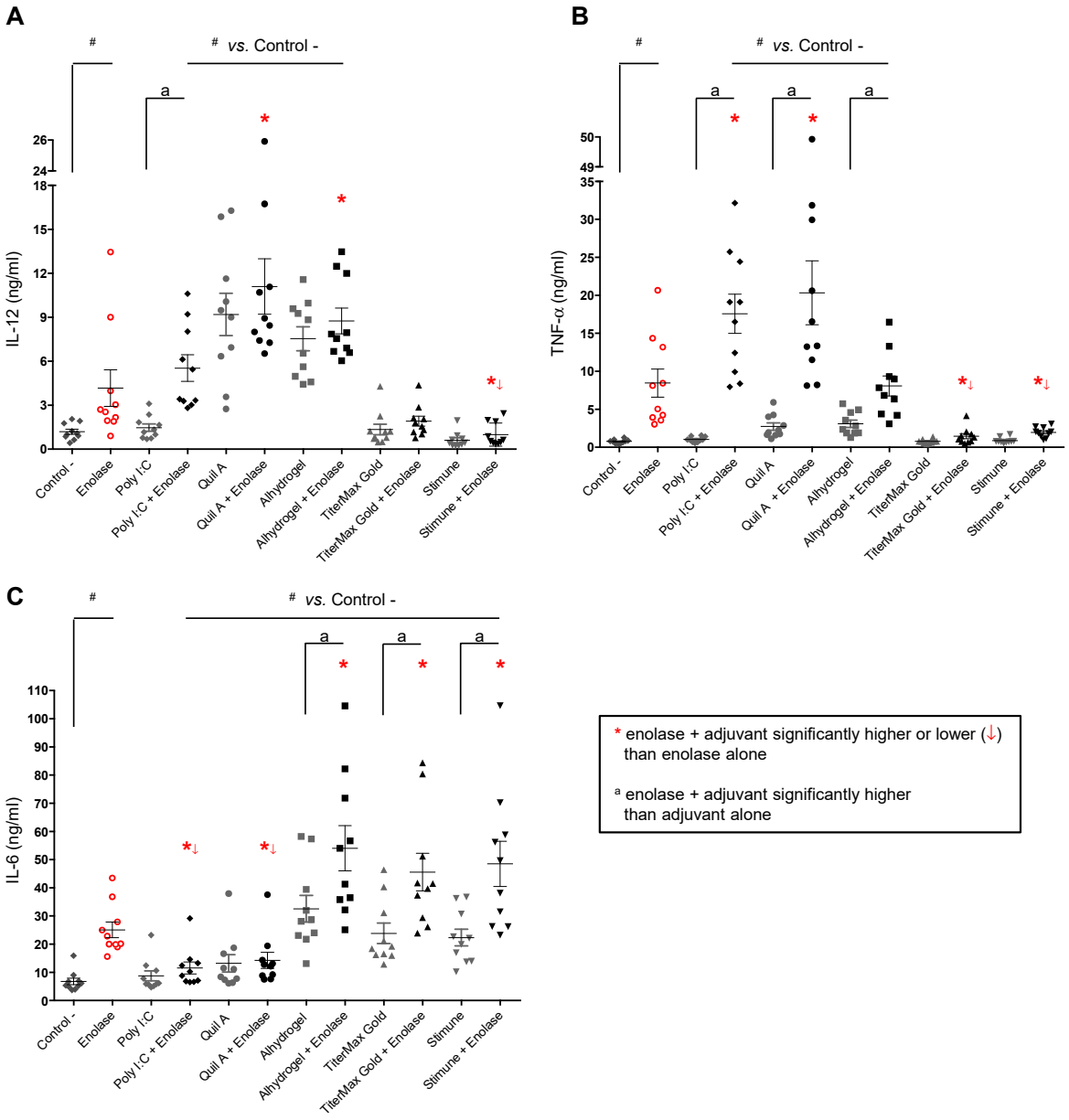


Figure 1. Cytokine production by bmDCs is differentially modified by enolase in combination with different adjuvants.

BmDCs derived from 10 different animals were incubated with enolase (50 µg/mL – final concentration for all conditions), alone or in combination with the adjuvants Poly I:C (50

μg/mL), Quil A ® (5 μg/mL), Alhydrogel ® (50 μg/mL), TiterMax Gold ® (100 μL/well of adjuvant–antigen emulsion) or Stimune ® (100 μL/well of adjuvant–antigen emulsion). Adjuvants alone were also evaluated. Cells incubated with medium served as negative controls (-). Cytokine levels (at 24 h of incubation) were evaluated by ELISA. Data of individuals are presented including mean ± SEM in ng/mL (n = 10). # $p < 0.0001$–0.0005, denotes values that are significantly higher than control (-). * $p < 0.0001$, denotes values obtained with enolase in combination with each adjuvant that are significantly higher or lower than enolase alone. a $p < 0.0001$–0.001 denotes values obtained with enolase in combination with each adjuvant that are significantly higher than the respective adjuvant alone.

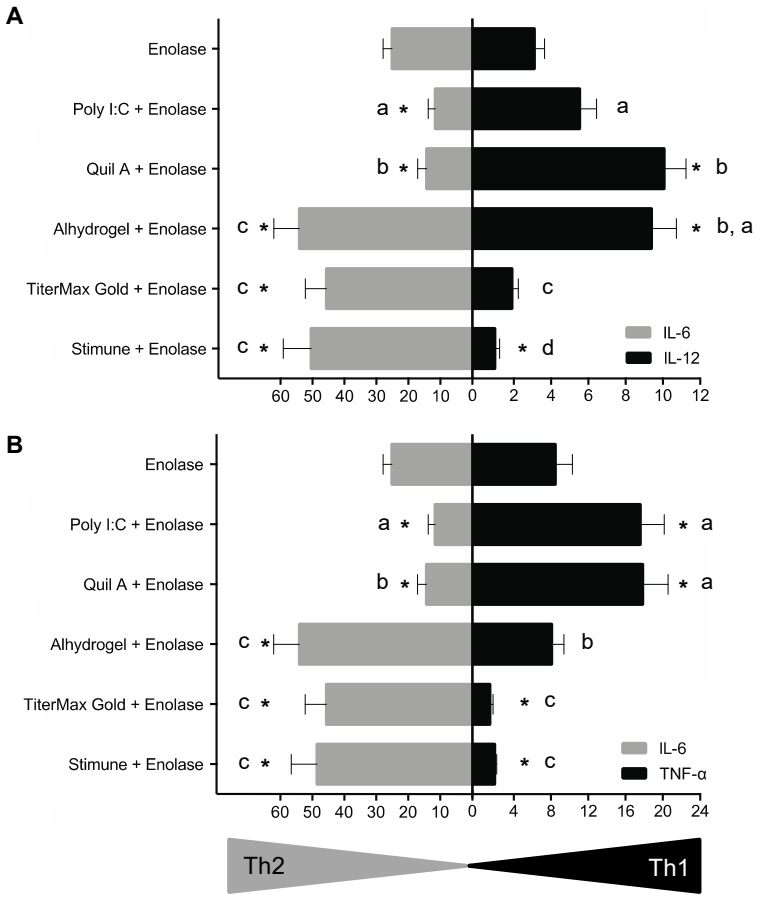


Figure 2. Type 1/type 2 cytokine profiles of bmDCs stimulated with enolase in combination with different adjuvants.

Data obtained in Figure 1 using bmDCs derived from 10 different animals and incubated with enolase (50 μg/mL – final concentration for all conditions), alone or in combination with Poly I:C (50 μg/mL), Quil A ® (5 μg/mL), Alhydrogel ® (50 μg/mL), TiterMax Gold ® (100 μL/well of adjuvant–antigen emulsion) or Stimune ® (100 μL/well of adjuvant–antigen emulsion) were analyzed using a linear mixed model to determine the polarizing effect of adjuvants. Data are expressed as mean ± SEM in ng/mL (n = 10). * $p < 0.0001$ denotes values obtained with enolase in combination with each adjuvant that are significantly higher or lower than enolase alone. Letters indicate differences between adjuvants in their capacity to induce the different cytokines ($p < 0.0001$–0.0005).

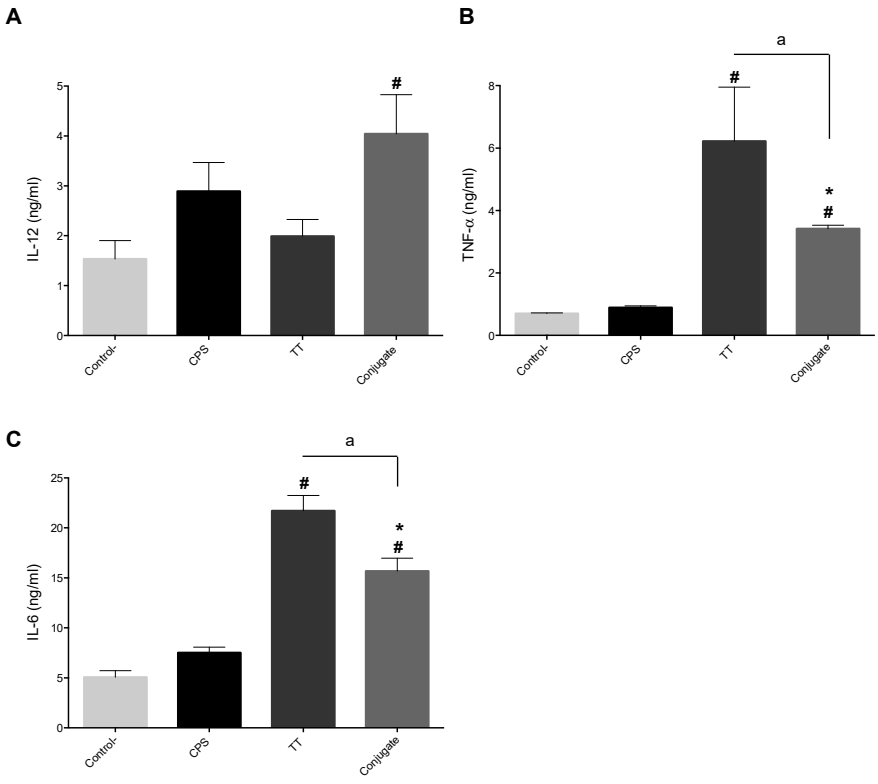


Figure 3. Cytokine production by bmDCs in response to stimulation by capsular polysaccharide (CPS), tetanus toxoid protein (TT) or CPS-TT conjugate.

BmDCs (from three different animals) were incubated with CPS, TT, or CPS-TT conjugate at 25 μg/mL. Cells incubated with medium served as negative controls (-). Cytokine levels (at 24 h of incubation) were evaluated by ELISA. Data are expressed as mean ± SEM in ng/mL (n = 3). # $p < 0.05$ denotes values that are significantly higher than control (-).* $p < 0.01$, denotes values obtained with CPS-TT conjugate that are significantly higher than CPS alone. a $p < 0.05$ denotes values obtained with CPS-TT conjugate that are significantly lower than TT alone.

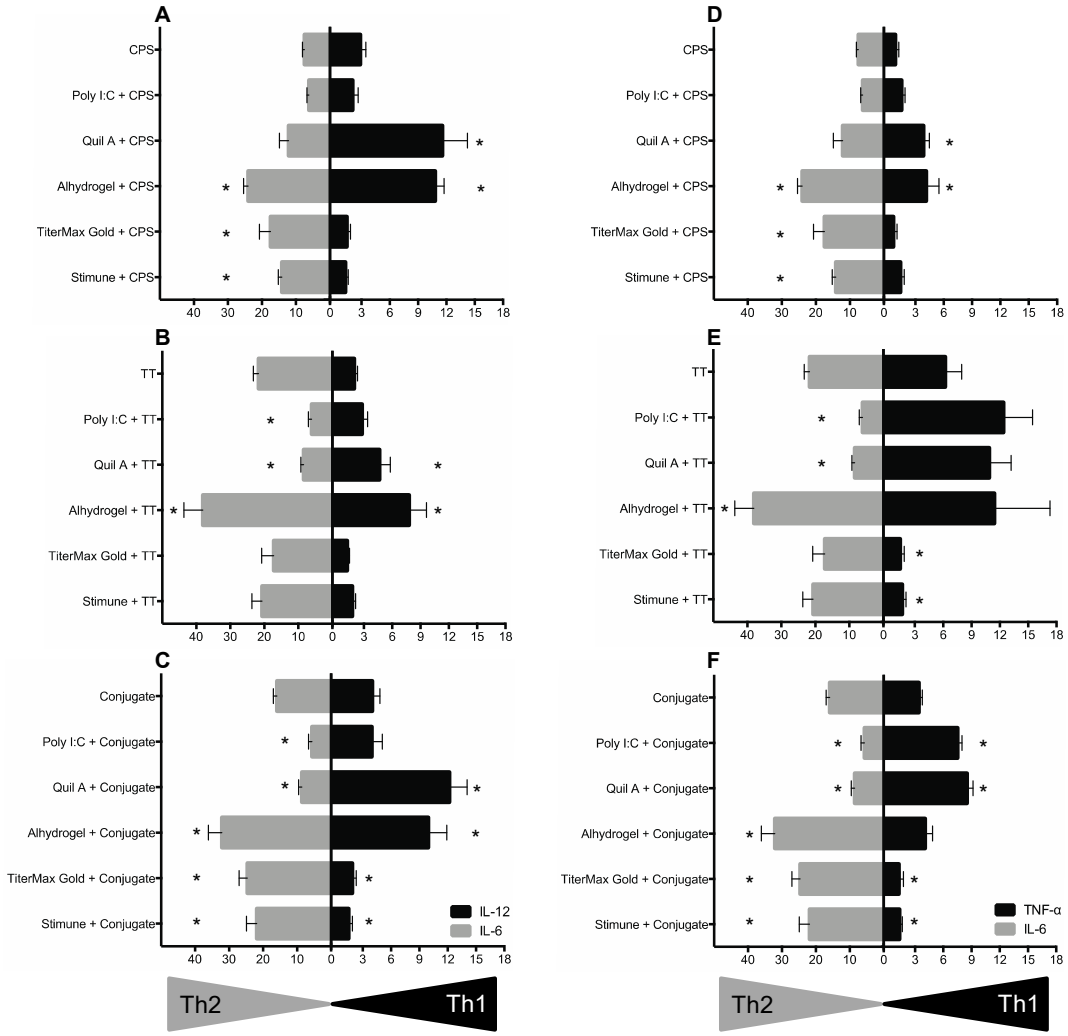


Figure 4. Type 1/type 2 cytokine profiles of bmDCs stimulated with antigens of diverse chemical natures in combination with different adjuvants.

BmDCs (from 3 different animals) were incubated with capsular polysaccharide (CPS), tetanus toxoid protein (TT), or CPS-TT conjugate (25 μg/mL – final concentration for all conditions), alone or in combination with the adjuvants Poly I:C (50 μg/mL), Quil A ® (5 μg/mL), Alhydrogel ® (50 μg/mL), TiterMax Gold ® (100 μL/well of adjuvant–antigen emulsion) or Stimune ® (100 μL/well of adjuvant–antigen emulsion). Cytokine levels (at 24 h of incubation) were evaluated by ELISA. Data are expressed as mean ± SEM in ng/mL (n = 3). * $p < 0.05$ denotes values obtained with CPS, TT or conjugate in combination with each adjuvant that are significantly higher or lower than the antigen alone.

Supporting information

Supplementary material is available with the article through the journal Web site at https://www.mdpi.com/2076-0817/6/1/13#supplementary.

Figure S1. Dose-response and toxicity trials to select optimal adjuvant dose for in vitro bmDC studies.

Figure S2. Dose-response to select optimal enolase concentration for in vitro bmDC studies.

ANNEXES - ARTICLE XVII

Glycoengineered Outer Membrane Vesicles: A Novel Platform for Bacterial Vaccines

---

Nancy L. Price [1], **Guillaume Goyette-Desjardins** [2], Harald Nothaft [1], Ezequiel Valguarnera [3], Christine M. Szymanski [1], Mariela Segura [2], and Mario F. Feldman [1,3]

[1] Department of Biological Sciences, University of Alberta Edmonton, Alberta T6G 2E9 Canada

[2] Laboratory of Immunology, Faculty of Veterinary Medicine, University of Montreal Saint-Hyacinthe, Quebec, J2S 2M2 Canada

[3] Department of Molecular Microbiology, Washington University School of Medicine, St Louis, MO 63110, USA

Publié dans *Scientific Reports*. **2016**. 6:24931.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé activement à la réalisation des expériences (opsonophagocytose; 20%) et à l'analyse des résultats.

Abstract

The World Health Organization has indicated that we are entering into a post-antibiotic era in which infections that were routinely and successfully treated with antibiotics can now be lethal due to the global dissemination of multidrug resistant strains. Conjugate vaccines are an effective way to create a long-lasting immune response against bacteria. However, these vaccines present many drawbacks such as slow development, high price, and batch-to-batch inconsistencies. Alternate approaches for vaccine development are urgently needed. Here we present a new vaccine consisting of glycoengineered outer membrane vesicles (geOMVs). This platform exploits the fact that the initial steps in the biosynthesis of most bacterial glycans are similar. Therefore, it is possible to easily engineer non-pathogenic *Escherichia coli* lab strains to produce geOMVs displaying the glycan of the pathogen of interest. In this work we demonstrate the versatility of this platform by showing the efficacy of geOMVs as vaccines against *Streptococcus pneumoniae* in mice, and against *Campylobacter jejuni* in chicken. This cost-effective platform could be employed to generate vaccines to prevent infections caused by a wide variety of microbial agents in human and animals.

Introduction

Most successful current antibacterial vaccines are glycoconjugates, composed of cell surface carbohydrates chemically attached to an appropriate carrier protein. These are effective means to generate protective immune responses to prevent a wide range of diseases. One of the best examples is the conjugate vaccine against *Haemophilus influenzae* type b, which practically eliminated the disease caused by this bacterium in vast parts of the world. Other examples are the vaccines against *Streptococcus pneumoniae* and *Neisseria meningitidis*, both based on capsular polysaccharides[1,2]. However, the current technology to produce conjugate vaccines presents some drawbacks[3,4,5]. These require complex synthetic chemistry for obtaining, activating, and attaching the polysaccharides to protein carriers. The carbohydrates employed for the formulation of these vaccines are usually obtained from pathogenic organisms, which may constitute a hazard and may require higher levels of biosafety for production. Some of the glycans containing acid-labile sugars do not resist the chemical treatment required for their purification and crosslinking to proteins. Furthermore, the chemical crosslinking is often not reproducible, resulting in unreliable products with batch to batch variability. Novel approaches are needed to effectively prevent bacterial infections, especially in the context of the alarming increase of MDR bacterial strains.

Gram-negative bacteria are able to produce outer membrane vesicles (OMVs). OMVs are mainly composed of LPS, outer membrane and periplasmic proteins, and phospholipids[6]. OMVs are formed by blebbing of the outer membrane, although their biogenesis is poorly understood[7]. Due to their immunogenic properties, self-adjuvanticity, ability to be taken up by mammalian cells, and capacity for enhancement by recombinant engineering, OMVs are attractive candidates for vaccine delivery platform[8]. Within the last 25 years, vesicle based meningococcal vaccines (i.e., MenBvac®, MeNZB®, and BexSero®) have been successfully developed and employed within various countries[9,10,11,12,13,14,15,16]. Due to their success, other OMV vaccine candidates for various pathogenic bacteria including *Vibrio cholerae*[17,18], *Bordetella pertussis*[19], *Burkholderia pseudomallei*[20,21], *Acinetobacter baumannii*[22] and even Gram-positive bacteria, such as *Bacillus anthracis*[23], have been tested. Such OMV based

vaccines involve direct manipulations of large volumes of pathogenic bacteria and may pack unwanted bacterial toxins.

Pneumococcal disease kills more patients worldwide than any other vaccine-preventable disease. Annual worldwide statistics show that 1.6 million people die of pneumococcal disease[24]. The current vaccine (Prevnar 13®) protects against the 13 predominant serotypes in the USA, but serotypes that are important in other countries are not included in the vaccine. Furthermore, serotypes with lower prevalence often take over the niche left by the serotypes included in the vaccines[25]. Therefore effective vaccines for the remaining S. pneumoniae capsule serotypes are needed. *Campylobacter jejuni* is a natural commensal in all birds, including chickens. Humans are frequently infected with *C. jejuni* through the consumption of improperly cooked poultry. In most cases, infection only causes diarrhea, fever and abdominal pain. However, approximately, one in one thousand patients will develop a severe polyneuropathy known as Guillain-Barré syndrome (GBS)[26]. One way to control human infection by *C. jejuni* would be to reduce the load of the bacterium in chickens. Here we present an alternative platform for vaccines consisting of glycoengineered OMVs (geOMVs) derived from non-pathogenic engineered *Escherichia coli* strains expressing bacterial surface glycans encoded by the bacterial pathogenic organisms. As a proof of principle, we demonstrate the efficacy of geOMVs as vaccines for *S. pneumoniae* serotype 14, and the common foodborne pathogen *C. jejuni*.

Results

Generation of geOMV displaying S. pneumoniae serotype 14 capsule (CPS14)

The general strategy for the production of geOMV is represented in Fig. 1. In principle, our platform can be applied to any surface glycan, such as O antigens, capsules, exopolysaccharides, or glycans present in N- and O-glycoproteins. The initial steps in the biosynthesis of these glycans are common. These include the assembly of the glycan onto the undecaprenylpyrophosphate carrier and the flipping of the lipid-linked sugars across the inner membrane of Gram-negative or the cell membrane in Gram-positive bacteria. If these pathways are reconstituted in *E. coli*, the WaaL ligase transfers the glycan from the carrier to

the lipid A, which is synthesized independently, and exported to the bacterial surface. OMVs displaying such antigens are then naturally produced. The biosynthetic pathways of type I capsular polysaccharides and O antigens only diverge once the polysaccharide has been translocated across the inner membrane (Supplementary Data, Fig. S1a). WaaL is not glycan-specific and can transfer a variety of sugars onto the lipid A core[27]. We predicted that expressing the *S. pneumoniae* capsule in an *E. coli* strain lacking its own O antigen would result in an LPS consisting of capsular polysaccharide attached to the lipid A core. To obtain such a strain, we constructed an inducible plasmid (pNLP80) which expresses the capsule synthesis *cps* locus and includes all essential glycosyltransferases, the flippase (Wzx), and the polymerase (Wzy) required for the synthesis of the *S. pneumoniae* serotype 14 capsule (CPS14). The expression of CPS14 was evaluated in two different *E. coli* mutant strains, CLM37[28] and CLM24[29] (Fig. 2a). CLM37 harbours a mutation which interrupts the initiator glycosyltransferase (wecA) for O antigen and Enterobacterial common antigen (ECA) synthesis[28]. In *Streptococcus* capsule synthesis the initiating glycosyltransferase is WchA, which transfers a glucose residue to the Und-PP carrier. WecA and WchA attach different sugars to the lipid and therefore would compete for the carrier. We reasoned that a wecA mutant strain, such as CLM37, in which competition for Und-PP is minimized, should produce more CPS14 than the wild-type strain. To demonstrate the attachment of the CPS14 to lipid A, we employed strain CLM24 (waaL mutant)[29]. In this strain the glycan remains attached to the lipid carrier and CPS14 should not be transferred to the lipid A. LPS extractions and OMV obtained from the two *E. coli* mutants show the presence of CPS14 in the WecA mutant but not in the WaaL ligase mutant strain, which indicates that CPS14 is attached to the lipid A core and directed to the vesicles in strains such as CLM37 that carry an intact WaaL (Fig. 2a). The signal detected in the WaaL mutant strain corresponds to UndPP-linked CPS14. The geOMVs were visualized by transmission electron microscopy (TEM) (Fig. 2b).

geOMV displaying S. pneumoniae CPS14 raise specific antibodies and are effective in OPA assays

To determine whether geOMVs from CLM37 strain displaying the CPS14 would generate an immunogenic response in a murine model, we injected 2 μg of geOMVs per mouse (n = 10). Control groups immunized with either geOMVs lacking the capsule (empty

geOMVs) or the commercially available Prevnar 13®, were included for comparison. Booster doses of geOMVs and Prevnar® were administered on Days 14 and 28 and sera was collected weekly over a 42 day period. Collected sera of the immunized mice were assayed via Western blot analysis (Fig. 3a). Initial immunization with geOMVs did not elicit an IgG immunogenic response. However one week after the first booster dose (Day 21), high IgG titers in response to CPS14 were generated (Fig. 3a). The IgG response continued to be observed over the remaining course of the immunization schedule. To determine whether the immunogenic response was specific for serotype 14, we performed ELISAs with whole pneumococcal cells of serotypes 14 and 9 V (Supplementary Data, Fig. S1b,c). Sera from mice injected with geOMVs with CPS14 reacted only with *S. pneumoniae* serotype 14, thus demonstrating that the immunogenic response is specific for the glycoengineered serotype. Furthermore, at day 42, the levels of the IgG response of mice injected with pneumococcal geOMVs were comparable to the immunogenic response of mice injected with the commercially marketed pneumococcal vaccine Prevnar 13® (Fig. 3b) which suggested that the level of the protective effect could also be similar.

Opsonophagocytosis assays (OPA) are accepted as one the most reliable ways to evaluate the efficacy of pneumococcal vaccines[30]. OPA were performed to evaluate the efficacy of geOMVs against an *S. pneumoniae* serotype 14 infection. Day 42 sera obtained from the three groups of immunized mice (i.e., placebo geOMVs, pneumococcal geOMVs, and Prevnar 13®) were employed. OPA showed a significantly increased killing effect using pneumococcal geOMVs compared to placebo geOMVs (Fig. 4a), which suggests that isolated pneumococcal geOMVs elicit a protective effect in vitro. In addition, varying mouse serum concentrations (5% and 20%) showed a similar killing effect between pneumococcal geOMVs and the Prevnar 13 pneumococcal vaccine (Fig. 4b), thus demonstrating that geOMVs have a similar protective effect when compared to the commercially available Prevnar 13® vaccine.

geOMV reduce chicken colonization by C. jejuni

We next evaluated the use of geOMVs to vaccinate chickens against *C. jejuni*. We introduced a plasmid (pACYCpglBmut) that expresses the *C. jejuni* heptasaccharide N-glycan into *E. coli* EVV11. This strain carries a mutation in Wzy and therefore does not polymerize

the *C. jejuni* glycan as the wild-type strain does. We isolated geOMVs from this strain and analyzed them by Western-blot employing an N-glycan specific antibody. An immunoreactive signal migrating around 15 kDa that was absent in control OMVs clearly indicates that the *C. jejuni* N-glycan was displayed in geOMVs (Fig. 5a). To analyze the efficacy of the geOMVs containing the *C. jejuni* N-glycan, we tested the protective effect of these geOMVs in a *C. jejuni* chicken challenge model. Four groups of chickens (n = 8 for the positive control, n = 6 for the negative and experimental groups) were vaccinated orally with PBS, placebo geOMVs or *Campylobacter* geOMVs, and challenged with oral doses of PBS (negative control) or *C. jejuni* strain 81–176. Birds were monitored for 7 days before being euthanized and the amount of *C. jejuni* isolated from the cecum was determined for each bird. Birds that received PBS (Fig. 5b, group 2) and placebo geOMVs (Fig. 5b, group 3) showed similar levels of *C. jejuni* colonization after challenge. Chickens vaccinated with the N-glycan-containing geOMVs exhibited an almost 10^4-fold reduction in *C. jejuni* colonization after challenge when compared to the naïve and placebo groups (Fig. 5b, group 4). In agreement with this data, the IgY levels of chicken that received the geOMV containing the glycan were higher than the ones that received the empty OMV (Fig. S1d).

Discussion

Conjugate vaccines are an effective way to create a long-lasting IgG immune response. However, besides being expensive to produce, these vaccines present a series of drawbacks. Large volumes of pathogenic cells need to be cultured to obtain the polysaccharides and the chemical crosslinking of the sugars to the protein is complex, with large batch to batch variability. The development of novel conjugate vaccines is very slow. Diverse serotypes not included in Prevnar 13® are prevalently being isolated in different geographical locations, and therefore novel technologies that can accelerate the development of multivalent vaccines are required. The generation of conjugate vaccines through exploitation of bacterial protein glycosylation systems is a very promising alternative, but not all the glycan chains are efficiently attached to proteins by the oligosaccharyltransferase PglB, which requires a HexNAc residue at the reducing end of the sugar chain[31]. PglL, the N. meningitidis O-oligosaccharyltransferase, can recognize a galactose but not glucose residue at the reducing

end (Feldman, manuscript in preparation). Interestingly, about 90% of the *S. pneumoniae* strains contain glucose at the reducing end. Here we demonstrated that OMVs produced by glycoengineered *E. coli* expressing a glycan from unrelated bacterial pathogens, such as the CPS14 capsule from *S. pneumoniae* or the heptasaccharide derived from N-liked glycans from *C. jejuni*, can be effective in producing a significant immune response.

In the case of *S. pneumoniae*, geOMVs induced an immune response, as measured by serum IgG levels and efficacy in OPA tests, which was similar to the one generated by the most widely used commercial conjugate vaccine. In the future, a multivalent vaccine could be generated by mixing geOMVs carrying capsules from different serotypes. Furthermore, geOMVs could complement the current conjugate vaccines, especially for serotypes corresponding to glycan structures for which the conjugation has not been solved. Although the Western blots together with the specific antibody response to CPS14 and the OPA assays suggest that the right structure of the CPS14 has been displayed in the geOMVs, future work will confirm the exact structure attached to lipid A. In the case of the *C. jejuni* geOMV vaccine candidate, the 4 log reduction in chicken colonization is, to our knowledge, unprecedented for this microorganism. However, the power of the vaccine could be increased if, in addition to engineering *E. coli* to produce pathogenic bacterial surface glycans, the geOMVs were modified to express antigenic membrane proteins to be directed to the vesicles. Previous studies have already explored incorporating antigenic proteins from pathogenic species (*Neisseria*, *Streptococcus*, *Leishmania*, *Vibrio*, and *Yersinia*) into vesicles derived from laboratory *E. coli* and *Salmonella* strains[17,32,33,34,35]. Furthermore, OMV produced in *Salmonella* carrying pneumococcal protein antigens showed promise in murine models[36]. The geOMV platform would enable the glycans and proteins to synergistically increase the vaccine immunogenicity capacity. This can also be important for cases like in *S. pneumoniae*, in which a protein antigen could expand the protection to serotypes not included in the vaccines.

One of the main concerns with vesicle based vaccines is the safety issues as OMVs contain endotoxic LPS[37]. LPS lipid A has been shown to provoke severe/lethal inflammatory responses in the host[38,39,40]. The OMV vaccines employed in humans were derived from *Neisseria meningitidis*. Several studies have analyzed the effect of modifying the lipid A to abrogate its interactions to obtain OMVs with LPS preparations tailored for human vaccine

development[41,42,43]. For example, *N. meningitidis* strains lacking LpxM or LpxL also render a lipid A with minimal toxicity[41,44]. It has been shown that OMVs produced in *E. coli* can also be detoxified through modifications of the lipid A[37]. These modifications can be carried out through the action of lipid A deacylases, such as PagL[45]. The overexpression of *B. pertussis* PagL resulted in OMVs with lower endotoxic activity compared to wild type *B. pertussis* OMVs. Monophosphorylated lipid A species recently became the first new Food and Drug Administration-approved adjuvant in several decades[46]. Therefore, it might be possible to generate geOMVs with a perfect balance between reduced toxicity and optimal adjuvanticity by generating strains containing modifications in the levels of lipid A acylation, phosphorylation, and/or other modifications.

Material and Methods

Animal ethics statement

All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Welfare Committee of the University of Montreal (protocol #RECH-1523). Studies involving chickens were carried out in accordance with the protocol approved by the Animal Care and Use Committee at the University of Alberta using a 35 day challenge protocol.

Construction of pneumococcal glycan expression plasmid

The *cps* gene cluster responsible for the synthesis of *S. pneumoniae* capsule serotype 14, CPS14, (excluding the regulatory genes) was cloned via a 3-way ligation method. Two separate fragments of the CPS14 locus were amplified using a high fidelity polymerase (iProof™, BioRad). Fragment 1 amplified the wchA-wchM region using the forward primer (5′-ATAGAGCTCATGGATAAAAAAGGATTGGAAAT-3′) and reverse primer (5′-TTAAGAAATTCATCCTCATACAA-3′). Fragment 2 amplified the wchM-wciY region using the forward primer (5′-CTGGTCAACAAATATTAGAAAAA-3′) and reverse primer (5′ATACTCGAGATTCTTTCTGTAAACTCCAAAAA-3′). The underlined sequences denotes SacI and XhoI restriction enzyme sites, respectively. The PCR fragments 1 and 2 were

both designed to include the native HindIII site within the wchM gene, thus, after a HindIII RE digest, the two fragments were compatible and reconstituted the wchM gene after ligation. Both fragments were successfully amplified and digested with SacI/HindIII (fragment 1) or HindIII/XhoI (fragment 2) and cloned into the plasmid vector pBBR1MCS-2, which was double digested with SacI and XhoI, generating pBBR1MCS-CPS14. The cloned CPS14 locus was confirmed by restriction enzyme digests and sequencing. For proper expression of the CPS14 locus in *E. coli*, the CPS14 locus was subcloned into pWSK129. The wchA-wciY CPS14 fragment was cleaved from pBBR1MCS-CPS14 using SacI and XhoI restriction enzymes. pWSK129 was digested with SacI and SalI restriction enzymes. As XhoI and SalI generate compatible sticky ends, the wchA-wciY fragment was ligated into pWSK129. The resulting plasmid, named pNLP80 was introduced in *E. coli* strains.

Isolation of geOMVs from E. coli expressing bacterial glycans

geOMVs were isolated following the protocol described by Haurat et al.[47]. Briefly, *E. coli* strains harbouring plasmids expressing genes involved in glycan synthesis (i.e., pNLP80, pEQ3, pACYCpglBmut) and the respective vector control plasmids were grown at 37 °C overnight with shaking in 50 mL of Luria-Bertani (LB) broth plus appropriate antibiotics. The following day, each culture was subcultured (1:100 dilution) into 1 L LB or Terrific Broth (TB) plus antibiotics and grown at 37 °C with shaking for 2 h ($OD_{600} \sim 0.2$). Inductions of glycans were performed as follows: IPTG was added to a final concentration of 0.1 mM to induce expression of *Streptococcus* CPS14 (pNLP80) while the *Campylobacter* heptasaccharide biosynthetic gene cluster on pACYCpglBmut is constitutively expressed. The cultures were grown for an additional 26 h at 37 °C with shaking. After incubation, cells were harvested by centrifugation at 23,000 × g for 15 min and the supernatant was collected. The supernantants were filtered twice through 0.44 μm and 0.2 μm filters to remove any residual intact cells. The filtered supernatants were ultracentrifuged at 210,000 × g for 3 h at 4 °C. The geOMV pellets were collected and resuspended in buffered saline (PBS).

Analysis and quantification of geOMV

Presence of geOMVs containing *Streptococcus* CPS14 or *Campylobacter* heptasaccharide was confirmed by Western blotting. Quantification of geOMVs was

performed by measurement of KDO using a modified protocol described by Lee and Tasi[48]. Commercially available anti-rabbit CPS14 antibody (Statens Serum Institut, Denmark) was used as positive control (1:1000 dilution) to visualize the presence of the pneumococcal capsule.

Transmission electron microscopy

Isolated geOMVs were absorbed onto carbon-coated copper membrane grids for 3 min. Excess geOMVs were blotted away from membrane grid and samples were negatively stained with 2% (w/v) uranyl acetate (3 min). The grids were analyzed for presence of geOMVs using a Morgagni (FEI) transmission electron microscope (Biological Sciences Microscopy Facility, University of Alberta).

Murine studies with Pneumococcal geOMVs

Three groups of BALB/c mice (n = 10 per group, female, 4–6 weeks old, obtained from Charles River, WA) were immunized intraperitoneally. The test group was injected with 2 μg of geOMVs isolated from *E. coli* CLM37 strain expressing CPS14 (pNLP80). A placebo control group received geOMVs isolated from *E. coli* CLM37 strain not expressing CPS14. The third group was injected with commercially available dose of Prevnar 13 (500 μL as supplied). Sera were collected weekly via tail bleeds over a 42 day period and booster doses were administered on Days 14 and 28. Final bleed (Day 42) was via cardiac puncture. The presence of antibodies against CPS14 was analyzed by Western-blot using Odyssey imaging systems (LI-COR Biosciences, USA). geOMVs preparations digested with proteinase K were analyzed using 1:500 dilutions of the mouse sera.

Whole cell ELISAs

S. pneumoniae (serotype 9 V and 14, Statens Serum Institut, Denmark) was grown overnight on blood agar plates at 37 °C with 5% CO_2 aerobic conditions. The next day the cells were scraped from the agar plate, resuspended in 1× PBS, and heat inactivated by incubation at 60 °C for 2 h. The cells were diluted to $OD_{600} \sim 0.6$/mL in PBS with protease inhibitor and 100 μL were seeded into ELISA 96 well plates. The plates were incubated overnight at 4 °C. The following day, the wells were washed three times with 1× PBS before

blocking with 2.5% skim milk for 2 h. The wells were washed three times with PBS and incubated with 100 µL of mouse sera (1:500 dilution) was added to each well and the plates were incubated at room temperature for 1 h. After incubation, the wells were washed again three times with PBS and 100 µL of IgG anti-mouse-alkaline phosphatase antibody was added to each well and incubated at room temperature for 1 h. After the secondary antibody incubation, the wells were washed again three times with PBS and 100 µL of p-nitrophenyl phosphate was added to each well and the plates were incubated at 37 °C for 1 h followed by reading the absorbance at 405 nm on a BioTek™ plate reader.

Opsonophagocytosis Assay (OPA)

Blood collection

Blood was collected by intracardiac puncture from naïve female mice (Charles River, Wilmington, MA), treated with sodium heparin, then diluted to obtain 6.25×10^6 leukocytes/mL in RPMI 1640 supplemented with 5% heat-inactivated fetal bovin serum, 10 mM HEPES, 2 mM L-glutamine and 50 µM 2-mercaptoethanol. All reagents were from Gibco (Invitrogen, Burlington, ON, Canada).

Bacterial suspension preparation

Isolated colonies on sheep blood agar plates of *S. pneumoniae* serotype 14 (Statens Serum Institut, Denmark) were inoculated in 5 ml of Todd-Hewitt Broth (THB) (Becton Dickinson, Mississauga, ON, Canada) and incubated for 16 h at 37 °C with 5% CO_2. Working cultures were prepared by transferring 0.1 mL of 16 h-cultures into 10 mL of THB which was incubated for 5 h. Bacteria were washed 3 times and resuspended in PBS to obtain an OD_{600} value of 0.6, which corresponds to 2×10^8 colony forming units (CFU)/mL. Final bacterial suspension was prepared in complete cell culture medium to obtain a concentration of 6.25×10^4 CFU/mL. The number of CFU/mL in the final suspension was determined by plating samples onto Todd-Hewitt Agar (THA) using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA).

Assay

Diluted whole blood (5×10^5 total leukocytes) was mixed with 5×10^3 CFU *of S. pneumoniae* (MOI of 0.01) and either 5% or 20% (v/v) of serum from control (placebo) or vaccinated mice in a microtube to a final volume of 0.2 mL. Microtubes were incubated for 2 h or 4 h at 37 °C with 5% CO_2, with shaking. After incubation, viable bacterial counts were performed on THA using an Autoplate 4000 Automated Spiral Plater. Tubes with addition of naive mouse sera (5% or 20% v/v) or of commercial rabbit anti-*S. pneumoniae* type 14 serum (20% v/v) (Statens Serum Institut, Denmark), were used as negative and positive controls, respectively. The % of bacteria killed was determined using the following formula: % Bacteria killed = [1 − (bacteria recovered from sample tubes/ bacteria recovered from negative control tubes with naïve sera)] × 100. Final OPA conditions were selected based in several pre-trials using different incubation times and MOIs (data not shown). A more detailed procedure can be found in[49].

Chicken vaccination and challenge

In general each group contained up to 8 leghorn birds (Poultry Research Facility, University of Alberta) that were randomly tested for the presence of *Campylobacter* on the day of hatch (Day 1) by plating cloacal swabs onto selective Karmali agar. In all cases no *Campylobacter* colonies were observed after 48 h of incubation under microaerobic conditions at 37 °C. Vaccination was performed by orally gavaging with 300 µL of PBS containing 500 ng of geOMVs on Days 7 and 21. Control groups were gavaged with 300 µL of PBS only.

Birds were challenged on day 28 by oral gavaging with either PBS (negative control) or with 300 µL PBS containing 10^2 *C. jejuni* 81–176 cells. The challenge strain was prepared as follows: *C. jejuni* 81–176 was grown for 18 h on Mueller-Hinton (MH) agar and harvested with cold MH broth. Cells were washed twice with cold PBS and adjusted to an OD_{600} of 1.2 (OD_{600} of 1.2 equals 3×10^8 cell/mL). Serial dilutions in PBS were performed dependent on the final amount of cells that were administered. For example: 3×10^2 cells/mL [= 1×10^2 cells per 300 µL (=1 dose)]. Cells were maintained on ice until used. Birds were maintained for an additional 7 days after challenge and then euthanized. Ceca were removed and the contents were adjusted to 1 mg cecal content per 1 mL with sterile PBS. Aliquots of 10-fold

serial dilutions (in PBS) of the cecal contents were plated onto selective Karmali agar. CFU were determined after incubation of the plates for 48 h under microaerobic conditions.

ELISA for C. jejuni N-glycan-specific antibodies

Blood samples were collected on Day 28 (vaccine response prior to challenge) and kept at 37 °C until a firm blood clot was formed. Samples were centrifuged (5 min, 18.000 × g, 4 °C) and the supernatants (sera) were transferred to fresh tubes. After addition of glycerol to a final concentration of 10%, sera were stored at −20 °C until further use. For ELISA coating, *Campylobacter* N-glycan compounds (Cj-N-glycan) and their chemical conjugation to the protein carrier bovine serum albumin (BSA) was performed as described[50,51]. Maxisorb plates (Thermo Fisher) were coated with 500 ng of BSA-Cj-N-glycan conjugate per well for 18 h at 4 °C. After removal of unbound antigen the plate was blocked for 1 h at room temperature with PBS-Tween, 5% skim milk. After discarding the blocking solution 100 µL of chicken sera diluted 1:10 in PBS-Tween, 1% skim milk was added to each well. Plates were incubated for 1 h at room temperature and washed 3 times for 5 min with PBS-Tween. After addition of 100 µL of 2nd antibody solution (anti chicken IgY, diluted 1:500 in PBS-Tween, 1% skim milk) and incubation for 1 h at room temperature wells were washed 4-times for 5 min with 100 µL of PBS-Tween and developed using the 1-Step p-Nitrophenyl Phosphate (PNPP) assay following the instructions of the manufacturer (Thermo Fisher). Immuno-reactivity in each serum was determined after reading the plate at OD_{405} in a plate reader.

References

1. Levine O. S. et al. Global status of Haemophilus influenzae type b and pneumococcal conjugate vaccines: evidence, policies, and introductions. Curr Opin Infect Dis 23, 236–41 (2010).

2. Chavez-Bueno S. & Stull T. L. Pediatric vaccines on the horizon. Am J Med Sci. 340, 226–31 (2010).

3. Jones C. Vaccines based on the cell surface carbohydrates of pathogenic bacteria. An Acad Bras Cienc. 77, 293–324 (2005).

4. Seale A. & Finn A. What is the best way to use conjugate vaccines? Curr Opin Infect Dis 24, 219–24 (2011).

5. Bottomley M. J., Serruto D., Safadi M. A. & Klugman K. P. Future challenges in the elimination of bacterial meningitis. Vaccine 30 Suppl 2, B78–86 (2012).

6. Ellis T. N. & Kuehn M. J. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microbiol Mol Biol Rev 74, 81–94 (2010).

7. Bomberger J. M. et al. Long-distance delivery of bacterial virulence factors by Pseudomonas aeruginosa outer membrane vesicles. Plos Pathog 5, e1000382 (2009).

8. Acevedo R. et al. Bacterial outer membrane vesicles and vaccine applications. Front Immunol 5, 121 (2014).

9. Bjune G. et al. Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. Lancet 338, 1093–6 (1991).

10. Arnold R., Galloway Y., McNicholas A. & O'Hallahan J. Effectiveness of a vaccination programme for an epidemic of meningococcal B in New Zealand. Vaccine 29, 7100–6 (2011).

11. Holst J. et al. Vaccines against meningococcal serogroup B disease containing outer membrane vesicles (OMV): lessons from past programs and implications for the future. Hum Vaccin Immunother 9, 1241–53 (2013).

12. Fredriksen J. H. et al. Production, characterization and control of MenB-vaccine "Folkehelsa": an outer membrane vesicle vaccine against group B meningococcal disease. NIPH Ann 14, 67–79, discussion 79–80 (1991).

13. Haneberg B. et al. Intranasal administration of a meningococcal outer membrane vesicle vaccine induces persistent local mucosal antibodies and serum antibodies with strong bactericidal activity in humans. Infect Immun 66, 1334–41 (1998).

14. Feiring B. et al. Persisting immune responses indicating long-term protection after booster dose with meningococcal group B outer membrane vesicle vaccine. Clin Vaccine Immunol 13, 790–6 (2006).
15. Sierra G. V. et al. Vaccine against group B Neisseria meningitidis: protection trial and mass vaccination results in Cuba. NIPH Ann 14, 195–207, discussion 208–10 (1991).

16. Norheim G. et al. An outer membrane vesicle vaccine for prevention of serogroup A and W-135 meningococcal disease in the African meningitis belt. Scand J Immunol 76, 99–107 (2012).

17. Schild S., Nelson E. J., Bishop A. L. & Camilli A. Characterization of Vibrio cholerae outer membrane vesicles as a candidate vaccine for cholera. Infect Immun 77, 472–84 (2009).

18. Schild S., Nelson E. J. & Camilli A. Immunization with Vibrio cholerae outer membrane vesicles induces protective immunity in mice. Infect Immun 76, 4554–63 (2008).

19. Roberts R. et al. Outer membrane vesicles as acellular vaccine against pertussis. Vaccine 26, 4639–46 (2008).

20. Silva E. B. & Dow S. W. Development of Burkholderia mallei and pseudomallei vaccines. Front Cell Infect Microbiol. 3, 10 (2013).

21. Nieves W. et al. A naturally derived outer-membrane vesicle vaccine protects against lethal pulmonary Burkholderia pseudomallei infection. Vaccine 29, 8381–9 (2011).

22. McConnell M. J., Rumbo C., Bou G. & Pachon J. Outer membrane vesicles as an acellular vaccine against Acinetobacter baumannii. Vaccine 29, 5705–10 (2011).

23. Rivera J. et al. Bacillus anthracis produces membrane-derived vesicles containing biologically active toxins. Proc Natl Acad Sci. USA 107, 19002–7 (2010).

24. Centers for Disease, C. & Prevention. Progress in introduction of pneumococcal conjugate vaccine - worldwide, 2000–2012. MMWR Morb Mortal Wkly Rep 62, 308–11 (2013).

25. Lynch J. P. 3rd & Zhanel G. G. Streptococcus pneumoniae: epidemiology and risk factors, evolution of antimicrobial resistance, and impact of vaccines. Curr Opin Pulm Med. 16, 217–25 (2010).

26. Heikema A. P. et al. Campylobacter jejuni capsular genotypes are related to Guillain-Barre syndrome. Clin Microbiol Infect 21, 852 e1–9 (2015).

27. Heinrichs D. E., Monteiro M. A., Perry M. B. & Whitfield C. The assembly system for the lipopolysaccharide R2 core-type of Escherichia coli is a hybrid of those found in

Escherichia coli K-12 and Salmonella enterica. Structure and function of the R2 WaaK and WaaL homologs. J Biol Chem. 273, 8849–59 (1998).

28. Linton D. et al. Functional analysis of the Campylobacter jejuni N-linked protein glycosylation pathway. Mol Microbiol 55, 1695–703 (2005).

29. Feldman M. F. et al. Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in Escherichia coli. Proc Natl Acad Sci USA 102, 3016–21 (2005).

30. Romero-Steiner S. et al. Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. Clin Vaccine Immunol 13, 165–9 (2006). [PMC free article]

31. Wacker M. et al. Substrate specificity of bacterial oligosaccharyltransferase suggests a common transfer mechanism for the bacterial and eukaryotic systems. Proc Natl Acad Sci USA 103, 7088–93 (2006).

32. Holst J. et al. The concept of "tailor-made", protein-based, outer membrane vesicle vaccines against meningococcal disease. Vaccine 23, 2202–5 (2005). [PubMed]

33. Kesty N. C. & Kuehn M. J. Incorporation of heterologous outer membrane and periplasmic proteins into Escherichia coli outer membrane vesicles. J Biol Chem. 279, 2069–76 (2004).

34. Muralinath M., Kuehn M. J., Roland K. L. & Curtiss R. 3rd. Immunization with Salmonella enterica serovar Typhimurium-derived outer membrane vesicles delivering the pneumococcal protein PspA confers protection against challenge with Streptococcus pneumoniae. Infect Immun 79, 887–94 (2011). [PMC free article]

35. Schroeder J. & Aebischer T. Recombinant outer membrane vesicles to augment antigen-specific live vaccine responses. Vaccine 27, 6748–54 (2009).

36. Kuipers K. et al. Salmonella outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonization. Vaccine 33, 2022–9 (2015).

37. Zariri A. & van der Ley P. Biosynthetically engineered lipopolysaccharide as vaccine adjuvant. Expert Rev Vaccines 14, 861–76 (2015).

38. Raetz C. R. & Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem 71, 635–700 (2002).

39. Galanos C. et al. Endotoxic properties of chemically synthesized lipid A part structures. Comparison of synthetic lipid A precursor and synthetic analogues with biosynthetic lipid A precursor and free lipid A. Eur J Biochem 140, 221–7 (1984).

40. Galanos C., Rietschel E. T., Luderitz O. & Westphal O. Interaction of lipopolysaccharides and lipid A with complement. Eur J Biochem 19, 143–52 (1971).

41. van der Ley P. et al. Modification of lipid A biosynthesis in Neisseria meningitidis lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. Infect Immun 69, 5981–90 (2001).

42. Pupo E., Hamstra H. J., Meiring H. & van der Ley P. Lipopolysaccharide engineering in Neisseria meningitidis: structural analysis of different pentaacyl lipid A mutants and comparison of their modified agonist properties. J Biol Chem 289, 8668–80 (2014).

43. Arenas J. et al. Coincorporation of LpxL1 and PagL mutant lipopolysaccharides into liposomes with Neisseria meningitidis opacity protein: influence on endotoxic and adjuvant activity. Clin Vaccine Immunol 17, 487–95 (2010).

44. van de Waterbeemd B. et al. Improved OMV vaccine against Neisseria meningitidis using genetically engineered strains and a detergent-free purification process. Vaccine 28, 4810–6 (2010).

45. Asensio C. J. et al. Outer membrane vesicles obtained from Bordetella pertussis Tohama expressing the lipid A deacylase PagL as a novel acellular vaccine candidate. Vaccine 29, 1649–56 (2011).

46. Casella C. R. & Mitchell T. C. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. Cell Mol Life Sci 65, 3231–40 (2008).

47. Haurat M. F. et al. Selective sorting of cargo proteins into bacterial membrane vesicles. J Biol Chem 286, 1269–76 (2011).

48. Lee C. H. & Tsai C. M. Quantification of bacterial lipopolysaccharides by the purpald assay: measuring formaldehyde generated from 2-keto-3-deoxyoctonate and heptose at the inner core by periodate oxidation. Anal Biochem 267, 161–8 (1999).

49. Goyette-Desjardins G., Roy R. & Segura M. Murine Whole-Blood Opsonophagocytosis Assay to Evaluate Protection by Antibodies Raised Against Encapsulated Extracellular Bacteria. Methods Mol Biol 1331, 81–92 (2015).

50. Dwivedi R., Nothaft H., Reiz B., Whittal R. M. & Szymanski C. M. Generation of free oligosaccharides from bacterial protein N-linked glycosylation systems. Biopolymers 99, 772–83 (2013).

51. Nothaft H. et al. Diversity in the protein N-glycosylation pathways within the Campylobacter genus. Mol Cell Proteomics 11, 1203–19 (2012).

Figures

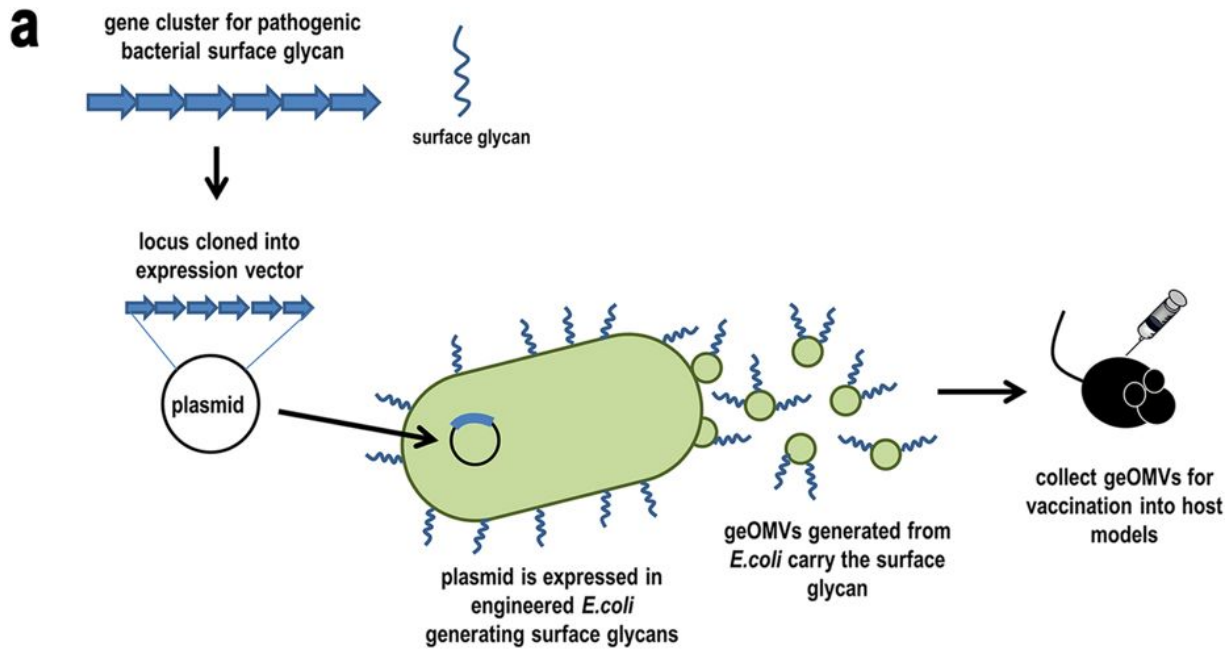


Figure 1. The geOMV platform.

Genetic locus encoding glycan structure of interest is cloned into expression vector and introduced into an engineered *E. coli* strain which generates vesicles that display the glycan. The collected geOMVs can be directly utilized for immunizations.

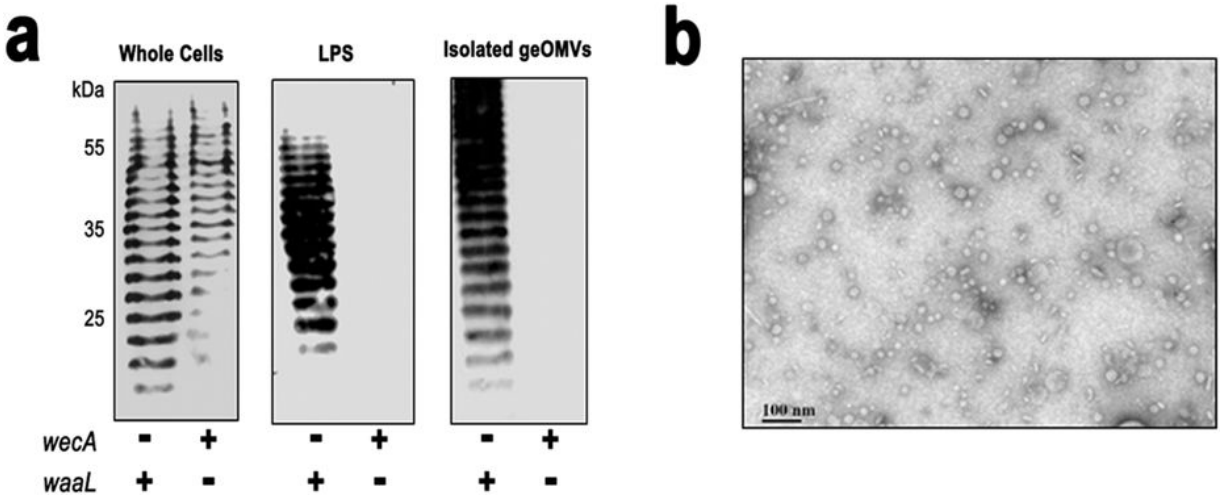


Figure 2. The *S. pneumoniae* serotype 14 capsule can be produced in *E. coli*.

(a) Western blot analysis of CPS14 expressed in *E. coli* strains CLM37 (wecA-; waaL+) and CLM24 (wecA+; waaL-) show the production of the pneumococcal capsule in whole cell lysates, LPS extractions, and isolated OMVs. (b) TEM of isolated pneumococcal geOMVs.

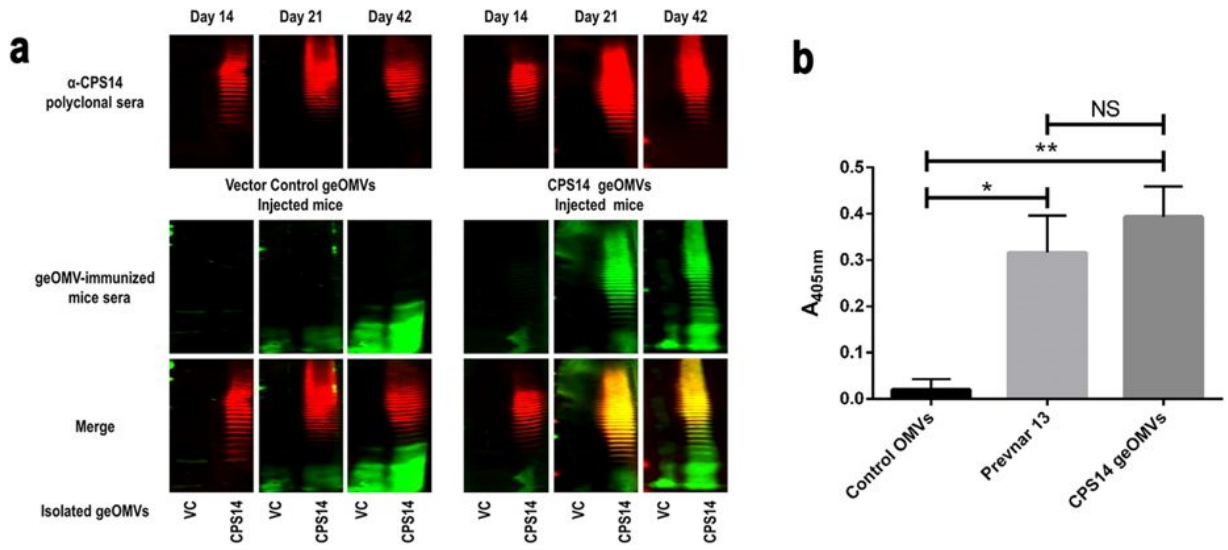


Figure 3. Sera from mice injected with geOMVs displaying *S. pneumoniae* serotype 14 capsule antigens cross react with the *S. pneumoniae* capsule.

(a) Western blots of geOMVs from CLM37 strain with vector control (VC) or *S. pneumoniae* serotype 14 capsule (CPS14). The *S. pneumoniae* serotype 14 capsule was visualized by either the commercially available rabbit anti-capsule serotype 14 antibody (red) and the mice sera (green). The overlapping signals are shown in yellow (merged). (b) Pneumococcal geOMVs display the similar immunogenic response as the commercial available Prevnar 13 pneumococcal vaccine. Mice sera (n = 10) collected at day 42 were incubated in wells of ELISA plates seeded with whole cell *S. pneumoniae* serotype 14. Figure shows statistically significant differences between means for each group. (*p-value < 0.0001; **p-value < 0.0001; NS p-value 0.0298). Error bars are SD and P values were calculated using the unpaired t-test.

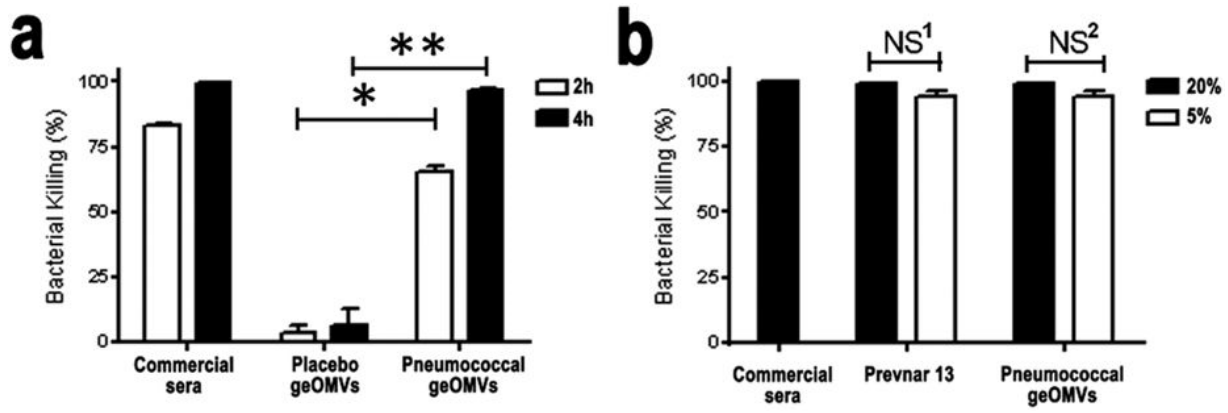


Figure 4. Bactericidal activity of sera from vaccinated mice against *S. pneumoniae* serotype 14.

(a) Opsonophagocytosis assay (OPA) evaluation of the effect of the incubation time (2 h and 4 h) on *S. pneumoniae* type 14 killing in presence of either 20% (v/v) placebo or vaccinated mice sera. Statistically significant differences were found at 2 hours and at 4 hours between the placebo and the vaccinated mice sera (*p-value < 0.0001; **p-value < 0.0001). Plotted data are means of percentages and SD as error bars. Kruskal-Wallis (non-parametric) test was used to calculate p-values. (b) OPA evaluation of the effect of the serum concentration (5% and 20%) on *S. pneumoniae* type 14 killing at 4 h incubation in presence of sera from mice vaccinated with either a pneumococcal geOMVs or Prevnar 13 commercial vaccine, used for comparative purposes. Commercial rabbit anti-*S. pneumoniae* type 14 serum was used as positive control to validate OPA methodology. No statistically significant differences could be observed between different serum concentrations (NS^1 p-value = 0.15; NS^2 p-value = 0.21). P-values were calculated using Kruskal-Wallis test. No bacteria were found when using 20% serum concentration.

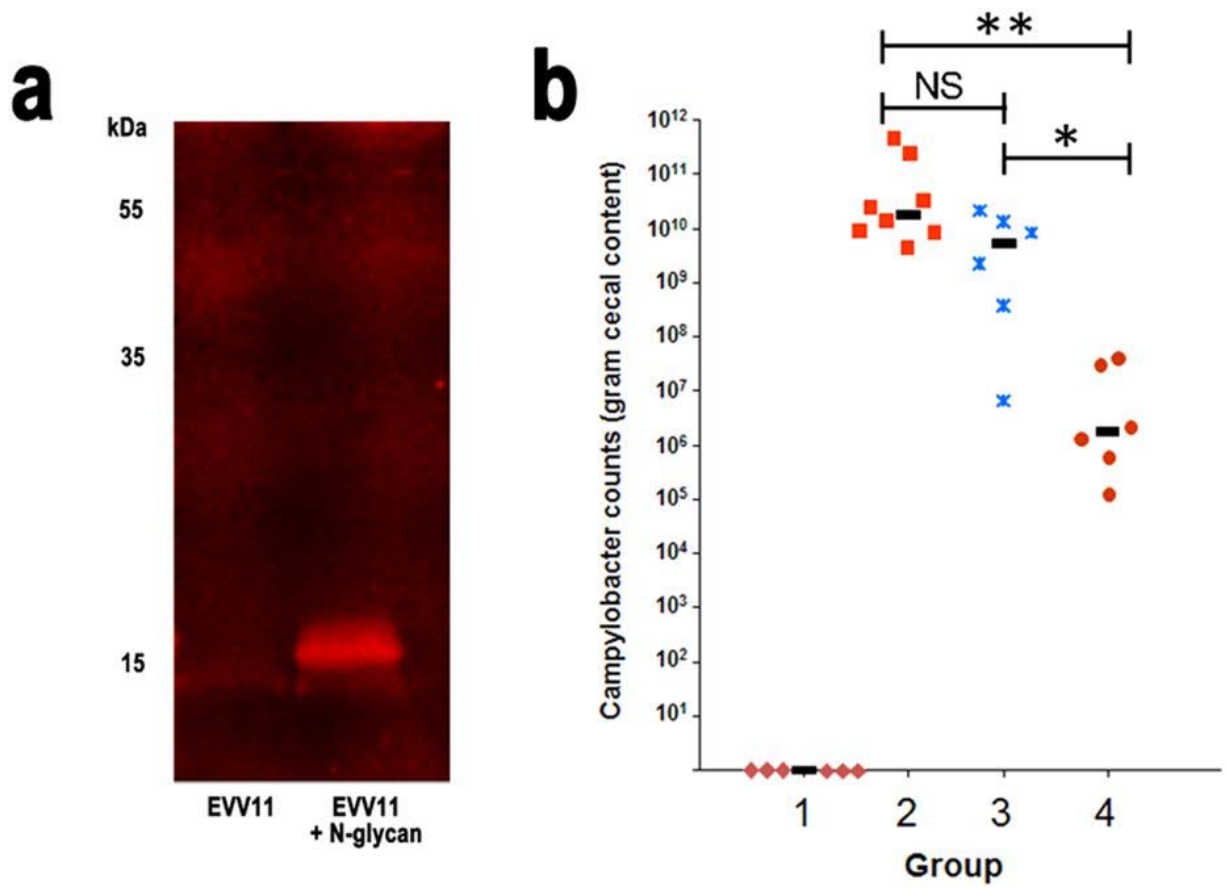


Figure 5. geOMVs displaying the N-heptasaccharide reduce *C. jejuni* colonization in chicken.

(a) Western blots of *E. coli* EVV11 geOMVs displaying no oligosaccharides (lane 1) and the heptasaccharide N-glycan (lane 2). (b) *Campylobacter* colonization in chicken. Group 1, non-vaccinated, not challenged (background control); group 2, non-vaccinated, challenged (colonization control); group 3, vaccinated with empty OMVs and challenged; group 4, vaccinated with geOMVs expressing the N-glycan and challenged. Each data point represents cfu/gram cecal content for one bird. The median for each group is represented by a black bar. No significant difference in colonization could be observed between groups 2 and group 3 (NS p-value = 0.29). A statistically significant reduction in colonization was observed in group 4 birds that received the Cj-N-glycan presenting OMVs when compared to either group 3 (*p-value = 0.045) or group 2 (**p-value = 0.032). No *Campylobacter* cells were detected in group 1, the detection limit based on the experimental setup was 200 cfu/gram cecal content. P-values were calculated by the one-way ANOVA test.

Supporting information

Supplementary material is available with the article through the journal Web site at https://www.nature.com/articles/srep24931#supplementary-information.

Figure S1. Supplementary Data.

ANNEXES - ARTICLE XVIII

A platform for glycoengineering a polyvalent pneumococcal bioconjugate vaccine using *E. coli* as a host

---

Christian M. Harding [1$], Mohamed A. Nasr [2$], Nichollas E. Scott [3], **Guillaume Goyette-Desjardins** [4], Harald Nothaft [2], Anne E. Mayer [5], Sthefany Chavez [5], Jeremy P. Huynh [5], Rachel L. Kinsella [5], Christine M. Szymanski [6], Christina L. Stallings [5], Mariela Segura [4], and Mario F. Feldman [1,5]

[$]These authors contributed equally to the manuscript

[1] VaxNewMo LLC, St. Louis, MO 63108, USA; [2] Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada T6G 2R3; [3] Department of Microbiology and Immunology, University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Parkville, VIC, 3010, Australia; [4] Swine and Poultry Infectious Diseases Research Center, Faculty of Veterinary Medicine, University of Montreal, 3200 Sicotte St., St-Hyacinthe, Quebec, J2S 2M2, Canada; Canadian Glycomics Network; [5] Department of Molecular Microbiology, Washington University School of Medicine, St Louis, MO 63110, USA; [6] Department of Microbiology and Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, USA

Publié dans *Nature Communications*. **2019**. 10(1): 891.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis coauteur de l'article. J'ai participé activement à la réalisation des expériences (opsonophagocytose; 30%) et à l'analyse des résultats.

Abstract

Conjugate vaccines, consisting of a polysaccharide linked to a protein, are lifesaving prophylactics. Traditionally, conjugate vaccines are manufactured using chemical methodologies. However, *in vivo* bacterial conjugations have emerged as manufacturing alternatives. *In vivo* conjugation (bioconjugation) is reliant upon an oligosaccharyltransferase to attach polysaccharides to proteins. Currently, the oligosaccharyltransferases employed for bioconjugations are not suitable for the generation of conjugate vaccines when the polysaccharides contain glucose at the reducing end. This limitation has enormous implications as ~75% of *Streptococcus pneumoniae* capsules contain glucose as the reducing end sugar. Here, we report the use of an *O*-linked oligosaccharyltransferase to generate the first ever polyvalent pneumococcal bioconjugate vaccine with polysaccharides containing glucose at their reducing end. Pneumococcal bioconjugates were immunogenic, protective and rapidly produced with recombinant techniques. These proof-of-principle experiments establish a platform to overcome limitations of other conjugating enzymes enabling the development of bioconjugate vaccines for many important human and animal pathogens.

Introduction

Streptococcus pneumoniae (pneumococcus) is a leading cause of bacterial induced pneumonia, meningitis, and bacteremia globally, particularly, afflicting children five years of age or younger(1, 2). Moreover, a 2000 epidemiological survey from the World Health Organization (WHO) estimated that 735,000 HIV-uninfected children died from pneumococcal related diseases(2) with updated estimates slightly reduced to 541,000 deaths for the year 2008 (ref.(3)). An increase in the number of prophylactic treatment options, mainly due to advancements in pneumococcal vaccine developments have emerged over the last two decades. Pneumovax®23, a 23-valent polysaccharide vaccine, is used in elderly populations as well as children over the age of two who are at increased risk of pneumococcal disease(4); however, polysaccharide vaccines typically act as T cell independent antigens and are generally not effective in children two years of age and younger(5). On the other hand, covalently linking a polysaccharide to a protein in the form of a conjugate vaccine elicits a T-cell dependent immune response across all age groups, characterized by high affinity IgG-producing plasma cells and memory B cells(6, 7).

Three pneumococcal conjugate vaccines have been commercially licensed since the year 2000: Prevnar®, Synflorix™, and Prevnar 13®. Prevnar 13®, the most broadly protecting pneumococcal conjugate vaccine, is comprised of 13 protein-polysaccharide conjugates consisting of pneumococcal serotypes 1, 3, 4, 5 ,6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F, each individually linked to the genetically inactivated diphtheria toxoid CRM_{197}. Although highly protective in a three-dose schedule, Prevnar 13® is one of the most expensive vaccines on the market today. This is mainly due to its complex manufacturing process resulting in a cost of ~$600 US dollars for primary and booster immunizations(8). In fact, Prevnar 13® has been Pfizer's best-selling product for the fiscal years 2015-2017 with total revenues exceeding 17.5 billion U.S. dollars(9). Although pneumococcal conjugate vaccines have significantly reduced the burden of pneumococcal disease events(10, 11), due to variations in global serotype distributions(12, 13), serotype replacement events(14), as well as the lack of a low-

cost alternative for developing countries, novel manufacturing strategies to expedite development of next generation vaccines are needed.

As mentioned above, currently licensed pneumococcal conjugate vaccines are synthesized chemically, which is a laborious process plagued with technical challenges, low yields, and batch-to-batch variations(15); highlighting the need for improved conjugate vaccine synthetic methodologies. Over the last 15 years, *in vivo* conjugation using bacterial protein glycosylation systems has emerged as a feasible alternative to chemical conjugation(16), with multiple bioconjugate vaccine candidates now in various stages of development and clinical trials(17, 18). Bioconjugation is based on exploiting protein glycosylation, a ubiquitous post-translational modification in which glycans are covalently linked to proteins. In bacteria, glycans are commonly bound to proteins via *N*- or *O*-linkages on asparagine or serine/threonine residues, respectively(19, 20). Several pathways for bacterial glycosylation have been characterized, and among the best described are the oligosaccharyltransferase (OTase)-dependent pathways in Gram negative bacteria(20). In these systems, a lipid-linked oligosaccharide is assembled sequentially at the cytoplasmic leaflet of the inner membrane, flipped to the periplasmic leaflet, and then transferred to acceptor proteins by either *N*- or *O*-OTases depending on the site of glycan attachment(20). Many bacterial species, including *S. pneumoniae*, also synthesize capsular polysaccharides employing the same lipid-linked oligosaccharides prior to their polymerization, export and transfer to the cell surface enabling their exploitation for bioconjugation reactions in *Escherichia coli*(21).

Glycoproteins have been recombinantly synthesized in *E. coli* for use as vaccines(16) and/or diagnostics(22, 23) by co-expressing three components: a genetic cluster encoding for the proteins required to synthesize a glycan of interest, an OTase and an acceptor protein. One drawback of this process is the apparent glycan substrate specificity of the known OTases, which, for some of them, has been suggested to be dictated by the reducing end sugar(24) (the first monosaccharide in the growing polysaccharide chain) of the lipid-linked oligo/polysaccharide of interest. Although OTases are able to transfer many different oligo- and polysaccharide structures(24, 25), some sugars have not been efficiently conjugated by

known OTases to acceptor proteins. Therefore, characterizing novel OTases is paramount for expanding our arsenal of therapeutic glycoproteins, including bioconjugate vaccines.

OTases currently used for commercially synthesizing glycoconjugates are the *Campylobacter jejuni N*-OTase PglB(16) and the *Neisseria meningitidis O*-OTase PglL(26), both of which exhibit a great deal of promiscuity towards glycan substrates(24, 25). However, neither enzyme has been experimentally demonstrated to conjugate glycans containing a glucose residue at the reducing end, such as ~75% of *S. pneumoniae* capsular polysaccharides (CPSs)(19, 27). In the present work, we demonstrate the first successful *in vivo* conjugation of *S. pneumoniae* CPSs containing glucose as the reducing end monosaccharide from multiple serotypes. This has been achieved using a new class of *O*-OTase, previously designated as $PglL_{ComP}$ by our group(28), and henceforth termed PglS. Here, we present proof of concept studies on the engineering, characterization, and immunological responses of a polyvalent pneumococcal bioconjugate vaccine using the natural acceptor protein ComP as a vaccine carrier as well as a monovalent pneumococcal bioconjugate vaccine using a conventional vaccine carrier containing the *Pseudomonas aeruginosa* exotoxin A protein.

Results

PglS, but not PglB or PglL, can conjugate pneumococcal CPS14 to proteins.

PglB, the first OTase described, was shown to preferentially transfer glycans containing an acetamido-group at the C-2 position of the reducing end sugar to asparagine residues of acceptor proteins(24). However, polysaccharides with galactose (Gal) at the reducing end, such as the *Salmonella enterica* Typhimurium O antigen, have been transferred by an engineered PglB variant(29) and also by PglL, the *O*-OTase from *N. meningiditis*(25). However, there is no evidence available for PglB or PglL mediated transfer of polysaccharides containing glucose (Glc) at the reducing end. We therefore tested the ability of PglB and PglL to transfer the pneumococcal CPS14, which has a Glc residue as the reducing end sugar, to their cognate acceptor proteins, AcrA and DsbA, respectively. As seen in Figure 1A-F, no evidence for CPS14 glycosylation to either acceptor protein was observed.

Previously we demonstrated that *Acinetobacter* species contain three *O*-linked OTases; a general PglL OTase responsible for glycosylating multiple proteins, and two pilin-specific OTases(28). The first pilin-specific OTase is an ortholog of TfpO (also known as PilO) and is not employed for *in vivo* conjugation systems due to its inability to transfer polysaccharides with more than one repeating unit(26). The second pilin specific OTase, PglS glycosylates a single protein, the type IV pilin ComP(28). A bioinformatic analysis indicated that PglS is the archetype of a distinct family of OTases, which prompted us to test its ability to transfer pneumococcal CPS14 to ComP. Western blotting analysis (Fig. 1G-I) showed that co-expression of the CPS14 biosynthetic locus in conjunction with PglS and a His-tagged variant of ComP resulted in a typical ladder-like pattern of bands compatible with protein glycosylation with multiple subunits. Both ComP-His (Fig. 1G) and CPS14 (Fig. 1H) were detected with antisera specific to each antigen; moreover, samples treated with proteinase K did not react with either the anti-His or anti-CPS14 antisera indicating that the purified material is indeed proteinaceous. Together, these results suggest that, unlike the previously characterized OTases, PglS is able to transfer polysaccharides with Glc at the reducing end.

Mass spectrometry and site directed mutagenesis confirm PglS is an O-linked OTase and reveal that ComP is glycosylated at a serine residue in position 84.

N-glycosylation in bacteria generally occurs within the sequon D-X-N-S-T, where X is any amino acid but proline(30). On the contrary, *O*-linked OTases do not seem to have defined recognition sequons. Most *O*-glycosylation events in bacterial proteins occur in regions of low complexity (LCR), rich in serine, alanine, and proline residues(31, 32). Some pilins are also *O*-glycosylated at a C-terminal serine residue(33). We were unable to find an obvious LCR or a C-terminal serine residue in ComP homologous to those found in other pilin like proteins and therefore employed mass spectrometry to determine the site(s) of glycosylation. Purified CPS14-ComP bioconjugates were subjected to GluC proteolytic digestion and multiple mass spectrometric analyses. As seen in Figure 2, we identified a single glycopeptide consisting of a semi-GluC derived peptide $_{81}$ISASNATTNVATAT$_{94}$ attached to a glycan that matched the published CPS14 composition (Fig. 2A). To enable the confirmation of both the peptide and attached glycan sequences, multiple collision energies regimes were performed to confirm the glycosylation of the semi-GluC derived peptide $_{81}$ISASNATTNVATAT$_{94}$ with a 1378.47 Da

glycan corresponding to $HexNAc_2Hexose_6$ (Fig. 2B). Additional glycopeptides were also observed decorated with extended glycans corresponding to up to four tetrasaccharide repeat units (Supplemental Figure 1).

We have previously shown that *Acinetobacter* species predominantly glycosylate proteins at serine residues and thus hypothesized that either serine 82 or 84 was the site of glycosylation(32). To determine which serine residue was the site of glycan attachment we employed the *C. jejuni* heptasaccharide as the donor glycan, due to the ease at which glycosylation is detectable from whole cell lysates. Wild type ComP was glycosylated with the *C. jejuni* heptasaccharide as indicated by its increased electrophoretic mobility and signal co-localization with hR6 anti-glycan sera when co-expressed with PglS (Supplemental Fig. 2A-C). MS analysis also confirmed the presence of the *C. jejuni* heptasaccharide on the identical semi-GluC derived peptide $_{81}$ISASNATTNVATAT$_{94}$ modified by CPS14 (Supplemental Fig. 3 and 4). As a negative control, we generated a catalytically inactive PglS mutant (H324A), which when co-expressed with the *C. jejuni* heptasaccharide glycan was unable to glycosylate wild type ComP (Supplemental Fig. 2A-C). We next performed site directed mutagenesis and observed that glycosylation of ComP with the *C. jejuni* heptasaccharide was abolished in the ComP[S84A] mutant, whereas ComP[S82A] was glycosylated at wild-type levels (Supplemental Fig. 2A-C). In addition, the site of ComP glycosylation was also determined using a pneumococcal polysaccharide and is discussed below.

Analysis of the immune responses to a monovalent CPS14-ComP bioconjugate vaccination in mice.

We evaluated the immunogenicity of a CPS14-ComP bioconjugate in a murine vaccination model. Two groups of mice (n=10) individually received 3 μg of either unglycosylated ComP or CPS14-ComP bioconjugate. Mice were boosted on days 14 and 28, and sacrificed on day 49 for whole blood collection. Each vaccine was formulated based on total protein. Using an enzyme linked immunosorbent assay (ELISA) with a serotype 14 strain of *S. pneumoniae* adsorbed to each well, we compared IgM and IgG responses to CPS14. As seen in Supplemental Figure 5, sera collected from mice vaccinated with a CPS14-ComP

bioconjugate had an increased IgG response specific to CPS14 (Supp. Fig.5B) but not an increased IgM response (Supp. Fig.5A). Further, we employed secondary HRP-tagged anti-IgG subtype antibodies to determine which of the IgG subtypes were present in CPS14-ComP vaccinated mice (Supp. Fig.5C). We determined that the CPS14 specific IgG1 response was higher than the other subtypes, which is consistent with previous findings for pneumococcal conjugate vaccines(34, 35).

Immunization with a pneumococcal tri-valent CPS8-, CPS9V-, and CPS14-ComP bioconjugate generated using PglS.

There are more than 90 serotypes of *S. pneumoniae*(21, 27). Many increasingly prevalent serotypes, like serotypes 8, 22F, and 33F are not included in currently licensed vaccines (36). Therefore, we tested the versatility of PglS to generate a multivalent pneumococcal bioconjugate vaccine against two serotypes included in Prevnar 13® (serotype 9V and 14) and one serotype not included (serotype 8). The aforementioned CPSs all contain Glc as the reducing end sugar and are therefore not compatible with other commercially exploited conjugating enzymes. As seen in Figure 3A-4F, western blot analyses of affinity purified proteins from whole cells co-expressing PglS, ComP, and either the CPS8 or CPS9V polysaccharides resulted in the generation CPS-specific ComP bioconjugates, respectively. Again, to confirm that the material purified was not contaminated with lipid-liked polysaccharides, we treated the samples with proteinase K and observed a loss of signal when analyzed via western blotting, confirming that the bioconjugates were proteinaceous.

Next, we performed a vaccination trial to determine the immunogenicity of a trivalent CPS8-, CPS9V-, and CPS14-ComP pneumococcal bioconjugate vaccine (Fig. 5A-5L). Three control groups were included, one group receiving carrier protein alone (unglycosylated ComP), another group receiving a monovalent vaccine of the CPS14-ComP bioconjugate to account for IgG specificity when analyzing immune responses against other serotypes, and a third group receiving Prevnar 13® as a positive control. All immunogen groups contained an equal mixture of Freund's adjuvant, including mice receiving Prevnar 13®. Day 49 sera from each group were analyzed by ELISAs on plates coated with *S. pneumoniae* serotypes 8, 9V

and 14. As mentioned above, serotypes 9V and 14 are included in Prevnar 13® and an elevated IgG response could be seen in Prevnar 13® immunized mice against these two serotypes 49 days post vaccination. Mice receiving the monovalent CPS14-ComP bioconjugate also showed significant IgG increase specific to serotype 14 specific (Fig. 5I). Mice receiving the trivalent CPS8-/CPS9V-/CPS14-ComP bioconjugate all had statistically significant increases in serotype specific IgG responses 49 days post vaccinations (Fig. 5J-5L).

Because Freund's adjuvant is not a suitable adjuvant for human clinical development, we performed another immunization trial with vaccines formulated with Imject Alum Adjuvant, a mild adjuvant containing a mixture of aluminum hydroxide and magnesium hydroxide. Vaccination cohorts included a buffer/adjuvant test group, a Prevnar 13® test group, and a trivalent CPS8-/CPS9V-/CPS14-ComP bioconjugate test group. Groups of three mice were vaccinated on days 1, 14, and 28. Serum was collected on day 42 and used to determine effector functions via an opsonophagocytosis assay (OPA). Given the limited amounts of sera collected from individual mice, sera were tested for bactericidal activity against serotypes 8 and 14, as one serotype is included in Prevnar 13® (serotype 14) and one is not (serotype 8). As seen in Figure 5A and 5B, serum from a representative mouse vaccinated with the trivalent CPS8-/CPS9V-/CPS14-ComP bioconjugate had increased bactericidal activity against *S. pneumoniae* serotype 14 strain when compared to sera from a mock vaccinated mouse. Importantly, that same bioconjugate vaccinated serum had high bactericidal activity against a *S. pneumoniae* serotype 8 strain, which was not observed for Prevnar 13® vaccinated sera due to the absence of this conjugate in its formulation.

Glycoengineering a pneumococcal bioconjugate with a conventional vaccine carrier.

Up to this point, we have exploited the use of ComP from *A. baylyi* ADP1 as a carrier protein for pneumococcal bioconjugate vaccine production; however, we sought to increase the commercial applicability of this technology by engineering a conventional vaccine carrier to be compatible with our *O*-linked OTase. To this end, we generated a chimeric fusion protein consisting of the ΔE553 variant of Exotoxin A from *Pseudomonas aeruginosa* (EPA) C-terminally fused to a ComP fragment lacking its first 28 amino acids (ComPΔ28). We used a ComP ortholog from *A. soli* strain 110264 (accession number ENV58402) as it was most

efficiently glycosylated by PglS and also found to be glycosylated at the same conserved serine as ComP from *A. baylyi* ADP1 (Supplemental Figure 6). The EPA fusion was linked to ComPΔ28 with a glycine-glycine-glycine-serine linker and trafficked to the periplasm with a DsbA signal sequence.

Because current formulations of pneumococcal conjugate vaccines do not contain a conjugate for serotype 8, we focused on generating an EPA-CPS8 pneumococcal bioconjugate. The EPA fusion was introduced into SDB1 cells co-expressing PglS and CPS8, subsequently purified, and then probed for glycosylation. As seen in Figure 5, the EPA fusion was efficiently glycosylated with CPS8. Furthermore, mass spectrometry analysis of intact glycoproteins confirmed that the EPA fusion was repetitively modified with an increasing mass unit of 662 Da, which is the calculated mass of a single CPS8 subunit. The EPA fusion was found to be glycosylated with at least 11 CPS8 subunits by intact protein analysis; however, western blot and Coomassie analyses indicated that >15 subunits were able to be transferred.

Subsequently, we performed an vaccination experiment comparing the immunogenicity of an EPA-CPS8 pneumococcal bioconjugate to a ComP-CPS8 pneumococcal bioconjugate. Groups of 10 mice were either vaccinated with 5 μg of EPA alone (based on total protein), 5 μg of ComP-CPS8 (based on polysaccharide as determined by anthrone sulfuric acid), or 100ng of EPA-CPS8 (based on polysaccharide as determined by mass spectrometry of intact EPA-CPS8). Mice were vaccinated on days 1, 14, and 28 with serum collected on day 42. All vaccinates were formulated 1:1 with imject Alum Adjuvant. ELISAs were subsequently performed to determine the IgG titers specific to CPS8. As seen in Figure 6A, mice vaccinated with either ComP-CPS8 or EPA-CPS8 has statistically significant increases in IgG titers specific to CPS8 when compared to EPA vaccinated mice. Additionally, the protective capacity of sera from vaccinated mice was determined using a murine adapted opsonophagocytosis assay (OPA) with whole blood leukocytes. As shown in Figure 6B, sera from vaccinated mice immunized with ComP-CPS8 displayed high levels of bactericidal killing ranging from 84- 50% with one mouse not displaying any killing activity. Moreover, sera from EPA-CPS8 vaccinated mice also displayed bactericidal ranging from 88%-10% with

three mice displaying no killing activity. Expectedly, sera from EPA vaccinate mice did not display killing activity.

Discussion

Traditional chemical conjugate vaccine synthesis is complex, costly, and laborious(15); therefore, new technologies to complement existing manufacturing pipelines are needed. One of these is bioconjugation, which has been thoroughly progressing as a feasible manufacturing alternative. The ability to glycosylate carrier proteins with polysaccharides containing Glc as the reducing end sugar has been elusive though, hindering the development of novel pneumococcal bioconjugate vaccines covering clinically relevant serotypes. Here we report, for the first time, the use of an *O*-linked OTase system for generating novel pneumococcal bioconjugate vaccines. Furthermore, we show that PglS naturally accepts polysaccharides containing Glc at the reducing end, a feat previously thought technically impossible due to substrate specificity limitations of all known conjugating enzymes.

The process of bioconjugation, described over a decade ago(16), has proven to be both technically and commercially feasible. This is best evidenced by the 2015 partnership between GlaxoSmithKline and GlycoVaxyn for more than $200 million USD. To date, bioconjugation relies on two conjugating enzymes, PglB and PglL, with much of the focus on PglB due to its inherent ability to glycosylate soluble proteins at a known sequon(30). As such, PglB has been the workhorse for the development and generation of bioconjugate vaccines in clinical trials. Examples include the Flexyn2a bioconjugate(18) against *Shigella dysenteriae* as well as a tetravalent ExPEc4V bioconjugate(17) vaccine against extraintestinal pathogenic *E. coli*. Recently, PglB was used to generate a bioconjugate vaccine against serotype 4 of pneumococcus(37). However, serotype 4 contains *N*-acetylgalactosamine as the reducing end sugar, which is one of the known substrates for PglB.

PglL also has commercially applicable features given its ability to transfer polysaccharides with galactose at the reducing end(25). However, until recently, PglL was thought to only glycosylate a few *Neisseria* proteins(31), most of which were membrane

associated proteins. Research by the Wang group though has resulted in the generation of a PglL specific sequon that can be engineered onto any carrier protein and efficiently be glycosylated by PglL, thus, rendering the production of PglL manufactured bioconjugates more practical(38, 39). However, PglB and PglL are not useful for the production of the overwhelming majority pneumococcal serotypes due to the presence of Glc at their reducing ends.

The genome of *A. baylyi* ADP1 encodes for two *O*-OTases, a PglL ortholog, which is a general OTase, and PglS, which glycosylates a single protein, ComP(28). ComP is orthologous to type IV pilin proteins, like PilA from *Pseudomonas aeruginosa* and PilE from *Neisseria gonorrhoeae,* both of which are glycosylated by the OTases TfpO (also known as PilO)(40) and PglL (also known as PglO)(41), respectively. TfpO glycosylates its cognate pilin at a C-terminal serine residue(33), which is not present in ComP. Some *Acinetobacter* strains also possess TfpO orthologs(28). PglL glycosylates PilE at an internal serine located at position 63 (ref. (42)). ComP contains serine residues near position 63 and the surrounding residues show limited conservation to PilE from *N. gonnorrhoeae*; however, Ser 63 and its surrounding residues were not part of the ComP glycosylation site. Instead, PglS glycosylates ComP at a single serine residue located at position 84, a novel glycosylation site which is not a canonical LCR, rich in proline, alanine, and serine residues. The ability of PglS to transfer polysaccharides containing Glc as the reducing end sugar coupled with the identification of a novel site of glycosylation within the pilin superfamily demonstrates that PglS is a functionally distinct OTase from PglL and TfpO, and suggest that PglS belongs to a new family of OTases.

Using the PglS/ComP OTase/acceptor protein pair, we have generated the first polyvalent pneumococcal bioconjugate vaccine and demonstrated its immunogenicity and efficacy using correlates of protection previously established as gold standards for pneumococcal conjugate vaccines(43). First, we demonstrate serotype specific IgG responses of CPS8-/CPS9V-/CPS14-ComP vaccinated mice. In these experiments, we found that the IgG response to all serotypes tested in bioconjugate vaccinated mice were robust as determined by ELISA. Second, we showed that serum from a mouse vaccinated with pneumococcal

bioconjugate vaccine was protective based on bactericidal killing assays against serotype 8 and 14 pneumococci. In addition, we have generated the first pneumococcal bioconjugate vaccine containing a conventional vaccine carrier. Namely, we have engineered the use of a ComP fragment as a glycotag, which can be added to the C-terminus of EPA. We then paired the EPA fusion with the CPS8 polysaccharide and PglS, generating the EPA-CPS8 bioconjugate, a first of its kind pneumococcal bioconjugate vaccine. The EPA-CPS8 bioconjugate vaccine elicited high IgG titers specific to serotype 8 specific that were protective as determined via bactericidal killing. Importantly, vaccination with as little as 100 ng of polysaccharide in the EPA-CPS8 bioconjugate was able to provide protection.

Even with the introduction and implementation of pneumococcal conjugate vaccines over the last two decades, hundreds of thousands of deaths are still attributed to pneumococcus each year (10). This is due in part to the 90+ serotypes of *S. pneumoniae* and the complex manufacturing methods required to synthesize pneumococcal conjugate vaccines. Together these factors hinder development of broader, more protective and less costly variations of the vaccines. Our bioconjugation platform for synthesizing pneumococcal conjugate vaccines from polysaccharides with Glc at the reducing end could expedite development and lower manufacturing costs. PglS-derived bioconjugates could complement existing manufacturing pipelines or completely bypass the dependency on chemical conjugation methodologies, enabling the production of a more comprehensive pneumococcal conjugate vaccine. Here we present data using the natural acceptor protein, ComP, as well as a proof of principle EPA fusion protein as the targets of PglS glycosylation. However, future iterations of the EPA vaccine construct will impart additional sites of glycosylation to increase the glycan to protein ratio as well as expand upon the number of serotypes in order to develop a comprehensive pneumococcal bioconjugate vaccine. Regardless, we present compelling data indicating that these pneumococcal bioconjugates have the potential for further commercial development. Importantly, the platform technology we present in this study is not limited to pneumococcal polysaccharides, but in fact, has vast applicability for generating bioconjugate vaccines for many important human and animal pathogens that are incompatible with PglB and PglL. Notable examples include the human pathogens *Klebsiella pneumoniae* and Group B

Streptococcus as well as the swine pathogen *S. suis*, all immensely relevant pathogens with no licensed vaccines available.

Material and Methods

Bacterial strains, plasmids and growth conditions.

Strains and plasmids used in this work are listed in Supplemental Table 1. Unless otherwise stated, *E. coli* strains were grown in Terrific Broth (TB) at 37°C overnight. *S. pneumoniae* strains were grown in Brain heart infusion (BHI) broth or sheep blood agar plates at 37°C in 5% CO_2. For plasmid selection the antibiotics were used at the following concentrations: ampicillin (100 μg/mL), tetracycline (20 μg/mL), chloramphenicol (12.5 μg/mL), kanamycin (20 μg/mL) and spectinomycin (80 μg/mL) were added as needed. Oligonucleotides used in this study are listed in Supplementary Table 2.

Heterologous glycosylation in E. coli.

Electrocompetent *E. coli* SDB1 was prepared as described by Dower and colleagues. Cells were electroporated with plasmids encoding the glycan synthesis loci, acceptor proteins and OTases. Colonies were picked and grown at 37°C in TB with appropriate antibiotic selection and immediately induced with 0.05-0.1 mM IPTG or 0.2% arabinose as needed and left overnight at 37°C. Cultures requiring arabinose induction received a second dose of arabinose after 4 hours. Cell pellets were obtained at stationary phases and prepared for western blot analysis.

Western blotting.

Cell lysates containing the equivalent of OD_{600} =0.1 units were loaded on 12.5% in-house prepared SDS-PAGE gels, which were then transferred to nitrocellulose membranes (Biorad). Western blots were employed to determine protein modification and antibodies used; concentrations and sources are outlined in Supplementary Table 2. Western blotting was performed according to our previously published protocols(28). Nitrocellulose membranes were then visualized using an Odyssey Infrared Imaging System (LiCor Biosciences, USA).

Purification of proteins and glycoproteins.

C-terminally Hexa-histidine-tagged ComP and ComP bioconjugates were purified from *E. coli* total membrane preparations. Cells were grown overnight in 2 L of terrific broth at 37°C, washed with phosphate buffered saline (PBS) buffer, and resuspended in 60 mL of the same buffer. Cells were lysed by two rounds of cell disruption at approximately 20 kPSI using a French press (Aminco) followed by the addition of a protease inhibitor cocktail (Roche). Lysates were centrifuged twice for 30 minutes at 20,000 x *g* to pellet cell debris. Supernatants were ultra-centrifuged at 100,000 x *g* for 60 mins to pellet total membranes. The pellets were resuspended in PBS buffer containing 0.5% n-dodecyl-β-D-maltoside (DDM) and membrane proteins were solubilized by tumbling for 48 hours. An equal volume of PBS was added to the suspension to reduce detergent concentration to 0.25% and the suspension was ultra-centrifuged at 100,000 x *g* for 60 mins. Solubilized membranes were filtered through 0.45µm and 0.22µm filters and loaded on a His-Trap HP column (GE Healthcare) fitted to an ÄKTA purifier (Amersham Biosciences, Sweden). The column was equilibrated with a PBS/DDM buffer containing 20 mM imidazole prior to loading the sample. Unbound proteins were removed by washing the column with seven column volumes of buffer containing 20mM and 30mM imidazole in PBS stepwise. To elute proteins bound to the column, a gradient elution with an incremental increase in imidazole concentration was used. The majority of unconjugated and conjugated ComP eluted between 180mM and 250mM imidazole. Imidazole was removed by an overnight round of dialysis followed by two 2-hour rounds through a 3.5 kDa dialysis membrane (Spectrum labs) in a 250mL dialysis buffer composed of PBS containing 0.25% w/v DDM. The final theoretical concentration of imidazole post dialysis was about 0.007 mM. Proteins were quantified using a DC kit (Biorad) after which the samples were diluted to the appropriate concentrations for mouse immunizations.

Murine model immunizations.

Immunizations were conducted at the Southern Alberta Cancer Research Institute (SACRI) antibody services and Washington University School of Medicine in St. Louis according to institutional guidelines. For the CPS14 ComP monovalent immunization, 4-6 week old female BALB/c mice were injected with 50 µL of purified protein/glycoprotein (3 µg total protein) with 50 µl of Freund's adjuvant. Two groups of mice (n=10) were injected

with either unglycosylated ComP (placebo) or CPS14-ComP bioconjugate. Sera from the mice were obtained before immunizations and 7, 21, 35 and 49 days post immunizations. Booster doses were given on days 14 and 28. The same procedure was followed for the trivalent immunization, except four groups of mice (n=10) were used for the four different immunization groups. These groups were injected with 100µL containing 3µg of unconjugated ComP (placebo) and Freund's adjuvant, 100µL containing 3 µg of ComP-CPS14 conjugate and Freund's adjuvant, 100µL containing 9µg of a glycoprotein mixture (ComP-CPS8, ComP-CPS9V and ComP-CPS14) and Freund's adjuvant, or 100µL of a 1:10 diluted stock of Prevnar 13® and Freund's adjuvant. CPS-ComP bioconjugates were formulated by total protein for this immunization.

Another trivalent immunization experiment was conducted with groups of three 4-6 week old female BALB/c mice. Each immunization group was subcutaneously injected with 100uL of a 1:1 immunogen (3 µg of protein of each of the trivalent bioconjugate or a 1:10 diluted stock of Prevnar 13®) to Imject Alum Adjuvant. Mice were vaccinated on day 0, 14, and 28 and then sacrificed on day 42 for sera collection.

A fourth immunization experiment was conducted with groups of three 4-6 week old BALB/c mice (five female and five male per group). Mice were immunized subcutaneously with 100 µL of EPA (5 µg total protein), 100 µL of ComP-CPS8 (5 µg total polysaccharide), or 100 µL of EPA-CPS8 (0.1 µg total polysaccharide) on day 0, 14, and 28 and then sacrificed on day 42 for sera collection. Vaccines were formulated 1:1 with Imject Alum Adjuvant.

Enzyme linked immunosorbent assays (ELISAs).

S. pneumoniae strains grown overnight in BHI broth at 37°C in 5% CO_2 were washed in PBS and the optical density was adjusted to OD_{600} =0.6 units. Cells were heat inactivated at 60°C for 2-4 hours followed by immobilization on high binding 96 well plates (Corning) by adding 50 µL/well. Plates were incubated on a tumbler overnight at 4°C. The following day, wells were washed three times with PBST (Phosphate buffered saline-tween) (100 µL/well) before blocking with 5% skimmed milk (250 µL/well) for 2 h. The wells were washed three times with PBST. Plates were incubated for 1 hour at room temperature with mouse sera (100

µL/well) at a 1:500 dilution in 2.5% skimmed milk in PBST. For the positive control, commercial rabbit polyclonal antibodies against CPS were used (Statens serum institute). Negative control wells were treated with skimmed milk without any primary antibody. After incubation with the primary antibody, wells were washed three times with PBST followed by a one hour incubation with secondary HRP-conjugated antibodies (100 µL/well) diluted in 2.5% skimmed milk in PBST. After incubation, the wells were washed three times with PBST and 100 µL of the chromogenic substrate TMB (Cell Signaling Technology) was added to each well. Plates were incubated at room temperature for 5 mins after which the absorbance at 650 nm was measured using a BioTek™ plate reader.

For IgG titer determinations, ELISA plates were coated with 100 µL of 1 x 10^8 CFU/mL of *S. pneumoniae* serotype 8 grown approximately to mid-log phase. Bacteria were washed twice in PBS and suspended in water prior to coating. ELISA plates were allowed to air dry in a biological hood for 24 hours. Fifty microliters of methanol were then added to each well and allowed to air dry. Plates were stored in a re-sealable bag protected from the light until use. To perform the titration of mouse total IgG antibodies, day 42 sera was serially diluted (2-fold) in PBST and antibodies were detected using an anti-mouse, HRP-linked IgG (Cell Signaling Technology # 7076) diluted 1:4000. For mouse serum titrations, the reciprocal of the last serum dilution that resulted in an optical density at 450nm equal to or lower than 0.2 was considered the titer of that serum. For representation purposes, negative titers (less than or equal to the cutoff) were given an arbitrary titer value of 10. Inter-plate variations were controlled by including an internal reference positive control on each plate. This control was hyper-immune sera from a mouse previously immunized with the ComP-CPS8 bioconjugate vaccine. The ELISA reactions in TMB were stopped when an OD450nm of ~1 was obtained for the internal positive control.

Site directed mutagenesis.

Mutagenic primers were designed using Primer X, a web-based primer design program (http://www.bioinformatics.org/primerx/). Primers used are listed in Supplementary Table 1. PCR reactions were performed using *Pfu* polymerase and 2-10 ng of pMN2 as template. The PCR reaction consisted of an initial denaturation of 30 s at 95˚c followed by 16 cycles of 30s

at 95˚C, 60s at 55˚C, 360s at 68˚C with no final extension. PCR reactions were DpnI digested for 2 hours to remove the template plasmid, then transformed into electrocompetent DH5α cells and grown on ampicillin for plasmid selection. Colonies were sequenced to confirm mutagenesis.

Digestion of ComP-CPS14 conjugate.

CPS14-ComP was affinity purified and separate via SDS-PAGE and Coomassie stained. SDS-PAGE separated CPS14-ComP bands were excised and destained in a 50:50 solution of 50 mM NH_4HCO_3 : 100% ethanol for 20 mins at room temperature with shaking at 750 rpm. Destained bands were then washed with 100% ethanol, vacuum-dried for 20 mins and rehydrated in 10 mM DTT in 50 mM NH_4HCO_3. Reduction was carried out for 60 mins at 56°C with shaking. The reducing buffer was then removed and the gel bands washed twice in 100% ethanol for 10 mins to ensure the removal of remaining DTT. Reduced ethanol washed samples were sequentially alkylated with 55 mM Iodoacetamide in 50mM NH_4HCO_3 in the dark for 45 mins at RT. Alkylated samples were then washed with 2 rounds of Milli-Q water and 100% ethanol then vacuum-dried. Alkylated samples were then rehydrated with 10 ng μl^{-1} GluC (Promega, Madison WI) in 40 mM NH_4HCO_3 at 4°C for 1 hour. Excess GluC was removed, gel pieces were covered in 40 mM NH_4HCO_3 and incubated for 24 hours at 37°C. Peptides were concentrated and desalted using C_{18} stage tips (44, 45) and stored on tip at 4°C. Peptides were eluted in buffer B (0.5% acetic acid, 80% MeCN) and dried before analysis by LC-MS.

Identification of glycopeptides using reversed phase LC-MS and HCD MS-MS.

Purified peptides were re-suspended in Buffer A* and separated using an in-house packaged 25 cm, 75 μm inner diameter, 360 μm outer diameter, 1.7 μm 130Å CSH C_{18} (Waters, Manchester, UK) reverse phase analytical column with an integrated HF etched nESI tip. Samples were loaded directly onto the column using an ACQUITY UPLC M-Class System (Waters) at 600 nL/min for 20 mins with Buffer A (0.1% FA) and eluted at 300 nL/min using a gradient altering the concentration of Buffer B (99.9% ACN, 0.1% FA) from 2% to 32% B over 60 mins, then from 32% to 40% B in the next 10 mins, then increased to

80% B over 8 mins period, held at 100% B for 2 mins, and then dropped to 2% B for another 10 mins. RP separated peptides were infused into a Q-Exactive (Thermo Scientific) mass spectrometer and data acquired using data dependent acquisition. Two methods were used to identify putative glycopeptides. Method A aimed to enable robust peptide identification in which one full precursor scan (resolution 70,000; 350-1850 m/z, AGC target of 1×10^6) was followed by 10 data-dependent HCD MS-MS events (resolution 35k AGC target of 1×10^5 with a maximum injection time of 110 ms, NCE 26 with 25% stepping) with 90 seconds dynamic exclusion enabled. Method B aimed to enable more complete characterization of glycans within glycopeptides with one full precursor scan (resolution 70,000; 350-1850 m/z, AGC target of 1×10^6) followed by 10 data-dependent HCD MS-MS events (resolution 35k AGC target of 5×10^5 with a maximum injection time of 250 ms, NCE 13 with 25% stepping) with 90 seconds dynamic exclusion enabled.

Database interrogation of identified glycopeptides.

Raw files were processed manually to identify potential glycopeptides based on the diagnostic oxonium 204.08 *m/z* ion. Putative glycopeptide derived scans were manually inspected and identified as possible GluC derived ComP glycopeptides based on the presence of an intense deglycosylated ComP derived peptide ion, matching within 10ppm using the Expasy FindPept tool (https://web.expasy.org/findpept/). To facilitate peptide assignments the resulting glycopeptides was manually annotated according to (46) with the aid of the Protein Prospector tool MS-Product (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct).

Intact Protein Analysis.

Intact analysis was performed using a 6520 Accurate mass Q-TOF mass spectrometer (Agilent, Santa Clara, CA). Protein samples were re-suspended in 2% acetonitrile, 0.1% TFA and immediately loaded onto a C5 Jupiter 5µm 300Å 50mm * 2.1 mm column (Phenomenex, Torrance, CA) Using an Agilent 1200. Samples were desalted by washing with buffer A (2% acetonitrile, 0.1% formic acid) for 4 mins and then separated with a 12 min linear gradient from 2 to 100% buffer B (80% acetonitrile, 0.1% formic acid) at a flow rate of 0.200ml/min.

MS1 Mass spectra were acquired at 1 Hz between a mass range of 300–3,000 *m/z*. Intact mass analysis and deconvolution was performed using MassHunter B.06.00 (Agilent).

Opsonophagocytosis Assay (OPA).

The assays were performed as previously described(47, 48) and are briefly described below. *Blood collection.* Blood was collected by intracardiac puncture from naïve female mice (Charles River, Wilmington, MA), treated with sodium heparin, then diluted to obtain 6.25×10^6 leukocytes/mL in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine and 50 μM 2-mercaptoethanol. All reagents were from Gibco (Invitrogen, Burlington, ON, Canada). *Bacterial suspension preparation.* Isolated colonies on sheep blood agar plates of either *S. pneumoniae* serotypes 8 or 14 (Statens Serum Institut, Denmark) were inoculated in 5 ml of Todd-Hewitt Broth (THB) (Oxoid, Thermo Fisher Scientific, Nepean, Canada) and incubated for 16 h at 37 °C with 5% CO_2. Working cultures were prepared by transferring 0.1 mL of 16 h-cultures into 10 mL of THB, which was then incubated for 5 h. Bacteria were washed 3 times and resuspended in PBS to obtain an OD_{600} value of 0.6, which corresponds to 1.5×10^8 and colony forming units (CFU)/mL and to 3.5×10^8 CFU/mL for serotype 8 and serotype 14, respectively. Final bacterial suspensions were prepared in complete cell culture medium to obtain a concentration of 6.25×10^4 CFU/mL. The number of CFU/mL in the final suspensions was determined by plating samples onto Todd-Hewitt Agar (THA). *Opsonophagocytosis Assay*. Diluted whole blood (5×10^5 total leukocytes) was mixed with 5×10^3 CFU of *S. pneumoniae* serotype 8 or 14 (MOI of 0.01) and 5% (v/v) of serum from control (placebo) or vaccinated mice in a microtube to a final volume of 0.2 mL. Microtubes were incubated for 4 h at 37 °C with 5% CO_2, with shaking. After incubation, viable bacterial counts were performed on THA. Tubes with the addition of naïve mouse sera or commercial rabbit anti-*S. pneumoniae* types 8 or 14 serum (Statens Serum Institut, Denmark), were used as negative and positive controls, respectively. The percent of bacteria killing was determined using the following formula: percent bacteria killed = [1 − (bacteria recovered from sample tubes / bacteria recovered from negative control tubes with naïve sera)] × 100.

Acknowledgments

We would like to thank Brendan Wren and Laura Yates for providing the pB-8 plasmid(49). We would like to thank Andrew Webb from Walter Eliza Hall institute of medical research for providing mass spectrometry support for the peptide-based analysis. We would also like to thank the Melbourne Mass Spectrometry and Proteomics Facility of The Bio21 Molecular Science and Biotechnology Institute at The University of Melbourne for the support, maintenance and access to mass spectrometry infrastructure for intact protein-based analysis. This work was funded by National Institute for Allergy and Infectious Disease (NIAID) R41 AI131742 grant. This work was partially supported by National Health and Medical Research Council of Australia (NHMRC) project grants awarded to NES (APP1100164). NES was supported by an Overseas (Biomedical) Fellowship (APP1037373) and a University of Melbourne Early Career Researcher Grant Scheme (Proposal number 603107). JPH was supported by a National Science Foundation Graduate Research Fellowship (DGE-1143954), RLK was supported by a Potts Memorial Foundation Postdoctoral Fellowship, and CLS was supported by a Burroughs Wellcome Fund Investigators in the Pathogenesis of Infectious Disease award.

References

1. Hausdorff WP, Hoet B, Adegbola RA. Predicting the impact of new pneumococcal conjugate vaccines: serotype composition is not enough. Expert Rev Vaccines. 2015;14(3):413-28.

2. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet. 2009;374(9693):893-902.

3. WHO. Estimated Hib and pneumococcal deaths for children under 5 years of age, 2008. 2013 [Available from: http://www.who.int/immunization/monitoring_surveillance/burden/estimates/Pneumo_hib/en.

4. CDC. Pneumococcal Disease 2017 [Available from: https://www.cdc.gov/pneumococcal/vaccination.html.

5. Cadoz M. Potential and limitations of polysaccharide vaccines in infancy. Vaccine. 1998;16(14-15):1391-5.

6. Avci FY, Li X, Tsuji M, Kasper DL. A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. Nat Med. 2011;17(12):1602-9.

7. Pollard AJ, Perrett KP, Beverley PC. Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. Nat Rev Immunol. 2009;9(3):213-20.

8. CDC. Vaccines for Children Program (VFC) 2018 [Available from: https://www.cdc.gov/vaccines/programs/vfc/awardees/vaccine-management/price-list/index.html.

9. Pfizer. Pfizer Inc. 2017 Financial Report 2017 [Available from: https://www.sec.gov/Archives/edgar/data/78003/000007800318000027/pfe-exhibit13x12312017x10k.htm.

10. Wahl B, O'Brien KL, Greenbaum A, Majumder A, Liu L, Chu Y, et al. Burden of Streptococcus pneumoniae and Haemophilus influenzae type b disease in children in the era of conjugate vaccines: global, regional, and national estimates for 2000-15. Lancet Glob Health. 2018;6(7):e744-e57.

11. Loo JD, Conklin L, Fleming-Dutra KE, Deloria Knoll M, Park DE, Kirk J, et al. Systematic review of the effect of pneumococcal conjugate vaccine dosing schedules on prevention of pneumonia. Pediatr Infect Dis J. 2014;33 Suppl 2:S140-51.

12. Grabenstein JD, Musey LK. Differences in serious clinical outcomes of infection caused by specific pneumococcal serotypes among adults. Vaccine. 2014;32(21):2399-405.

13. Cui YA, Patel H, O'Neil WM, Li S, Saddier P. Pneumococcal serotype distribution: A snapshot of recent data in pediatric and adult populations around the world. Hum Vaccin Immunother. 2017;13(6):1-13.

14. Gladstone RA, Jefferies JM, Tocheva AS, Beard KR, Garley D, Chong WW, et al. Five winters of pneumococcal serotype replacement in UK carriage following PCV introduction. Vaccine. 2015;33(17):2015-21.

15. Frasch CE. Preparation of bacterial polysaccharide-protein conjugates: analytical and manufacturing challenges. Vaccine. 2009;27(46):6468-70.

16. Feldman MF, Wacker M, Hernandez M, Hitchen PG, Marolda CL, Kowarik M, et al. Engineering *N*-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. Proc Natl Acad Sci U S A. 2005;102(8):3016-21.

17. Huttner A, Hatz C, van den Dobbelsteen G, Abbanat D, Hornacek A, Frolich R, et al. Safety, immunogenicity, and preliminary clinical efficacy of a vaccine against

extraintestinal pathogenic *Escherichia coli* in women with a history of recurrent urinary tract infection: a randomised, single-blind, placebo-controlled phase 1b trial. Lancet Infect Dis. 2017;17(5):528-37.

18. Riddle MS, Kaminski RW, Di Paolo C, Porter CK, Gutierrez RL, Clarkson KA, et al. Safety and Immunogenicity of a Candidate Bioconjugate Vaccine against *Shigella flexneri* 2a Administered to Healthy Adults: a Single-Blind, Randomized Phase I Study. Clin Vaccine Immunol. 2016;23(12):908-17.

19. Nothaft H, Szymanski CM. Protein glycosylation in bacteria: sweeter than ever. Nat Rev Microbiol. 2010;8(11):765-78.

20. Iwashkiw JA, Vozza NF, Kinsella RL, Feldman MF. Pour some sugar on it: the expanding world of bacterial protein *O*-linked glycosylation. Mol Microbiol. 2013;89(1):14-28.

21. Geno KA, Gilbert GL, Song JY, Skovsted IC, Klugman KP, Jones C, et al. Pneumococcal capsules and their types: past, present, and future. Clin Microbiol Rev. 2015;28(3):871-99.

22. Iwashkiw JA, Fentabil MA, Faridmoayer A, Mills DC, Peppler M, Czibener C, et al. Exploiting the *Campylobacter jejuni* protein glycosylation system for glycoengineering vaccines and diagnostic tools directed against brucellosis. Microb Cell Fact. 2012;11:13.

23. Ciocchini AE, Rey Serantes DA, Melli LJ, Iwashkiw JA, Deodato B, Wallach J, et al. Development and validation of a novel diagnostic test for human brucellosis using a glyco-engineered antigen coupled to magnetic beads. PLoS Negl Trop Dis. 2013;7(2):e2048.

24. Wacker M, Feldman MF, Callewaert N, Kowarik M, Clarke BR, Pohl NL, et al. Substrate specificity of bacterial oligosaccharyltransferase suggests a common transfer mechanism for the bacterial and eukaryotic systems. Proc Natl Acad Sci U S A. 2006;103(18):7088-93.

25. Faridmoayer A, Fentabil MA, Haurat MF, Yi W, Woodward R, Wang PG, et al. Extreme substrate promiscuity of the *Neisseria* oligosaccharyl transferase involved in protein *O*-glycosylation. J Biol Chem. 2008;283(50):34596-604.

26. Faridmoayer A, Fentabil MA, Mills DC, Klassen JS, Feldman MF. Functional characterization of bacterial oligosaccharyltransferases involved in *O*-linked protein glycosylation. J Bacteriol. 2007;189(22):8088-98.

27. Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, Collins M, et al. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet. 2006;2(3):e31.

28. Harding CM, Nasr MA, Kinsella RL, Scott NE, Foster LJ, Weber BS, et al. *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to *O*-glycosylation of multiple proteins. Mol Microbiol. 2015;96(5):1023-41.

29. Ihssen J, Haas J, Kowarik M, Wiesli L, Wacker M, Schwede T, et al. Increased efficiency of *Campylobacter jejuni N*-oligosaccharyltransferase PglB by structure-guided engineering. Open Biol. 2015;5(4):140227.

30. Kowarik M, Young NM, Numao S, Schulz BL, Hug I, Callewaert N, et al. Definition of the bacterial *N*-glycosylation site consensus sequence. EMBO J. 2006;25(9):1957-66.

31. Vik A, Aas FE, Anonsen JH, Bilsborough S, Schneider A, Egge-Jacobsen W, et al. Broad spectrum *O*-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*. Proc Natl Acad Sci U S A. 2009;106(11):4447-52.

32. Scott NE, Kinsella RL, Edwards AV, Larsen MR, Dutta S, Saba J, et al. Diversity within the *O*-linked protein glycosylation systems of *Acinetobacter* species. Mol Cell Proteomics. 2014;13(9):2354-70.

33. Comer JE, Marshall MA, Blanch VJ, Deal CD, Castric P. Identification of the *Pseudomonas aeruginosa* 1244 pilin glycosylation site. Infect Immun. 2002;70(6):2837-45.

34. Wuorimaa T, Dagan R, Vakevainen M, Bailleux F, Haikala R, Yaich M, et al. Avidity and subclasses of IgG after immunization of infants with an 11-valent pneumococcal conjugate vaccine with or without aluminum adjuvant. J Infect Dis. 2001;184(9):1211-5.

35. Soininen A, Seppälä I, Nieminen T, Eskola J, Käyhty H. IgG subclass distribution of antibodies after vaccination of adults with pneumococcal conjugate vaccines. Vaccine. 1999;17(15–16):1889-97.

36. Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J Infect Dis. 2010;201(1):32-41.

37. Herbert JA, Kay EJ, Faustini SE, Richter A, Abouelhadid S, Cuccui J, et al. Production and efficacy of a low-cost recombinant pneumococcal protein polysaccharide conjugate vaccine. Vaccine. 2018;36(26):3809-19.

38. Pan C, Sun P, Liu B, Liang H, Peng Z, Dong Y, et al. Biosynthesis of Conjugate Vaccines Using an *O*-Linked Glycosylation System. MBio. 2016;7(2):e00443-16.

39. Sun P, Pan C, Zeng M, Liu B, Liang H, Wang D, et al. Design and production of conjugate vaccines against *S. Paratyphi* A using an *O*-linked glycosylation system *in vivo*. NPJ Vaccines. 2018;3:4.

40. Castric P. *pilO*, a gene required for glycosylation of *Pseudomonas aeruginosa* 1244 pilin. Microbiology. 1995;141 (Pt 5):1247-54.

41. Aas FE, Vik A, Vedde J, Koomey M, Egge-Jacobsen W. *Neisseria gonorrhoeae O*-linked pilin glycosylation: functional analyses define both the biosynthetic pathway and glycan structure. Mol Microbiol. 2007;65(3):607-24.

42. Aas FE, Egge-Jacobsen W, Winther-Larsen HC, Lovold C, Hitchen PG, Dell A, et al. *Neisseria gonorrhoeae* type IV pili undergo multisite, hierarchical modifications with phosphoethanolamine and phosphocholine requiring an enzyme structurally related to lipopolysaccharide phosphoethanolamine transferases. J Biol Chem. 2006;281(38):27712-23.

43. Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol. 2010;17(7):1055-65.

44. Ishihama Y, Rappsilber J, Mann M. Modular stop and go extraction tips with stacked disks for parallel and multidimensional Peptide fractionation in proteomics. J Proteome Res. 2006;5(4):988-94.

45. Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nature protocols. 2007;2(8):1896-906.

46. Roepstorff P, Fohlman J. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed Mass Spectrom. 1984;11(11):601.

47. Goyette-Desjardins G, Roy R, Segura M. Murine Whole-Blood Opsonophagocytosis Assay to Evaluate Protection by Antibodies Raised Against Encapsulated Extracellular Bacteria. Methods Mol Biol. 2015;1331:81-92.

48. Price NL, Goyette-Desjardins G, Nothaft H, Valguarnera E, Szymanski CM, Segura M, et al. Glycoengineered Outer Membrane Vesicles: A Novel Platform for Bacterial Vaccines. Sci Rep. 2016;6:24931.

49. Kay EJ, Yates LE, Terra VS, Cuccui J, Wren BW. Recombinant expression of *Streptococcus pneumoniae* capsular polysaccharides in *Escherichia coli*. Open Biol. 2016;6(4):150243.

Figures

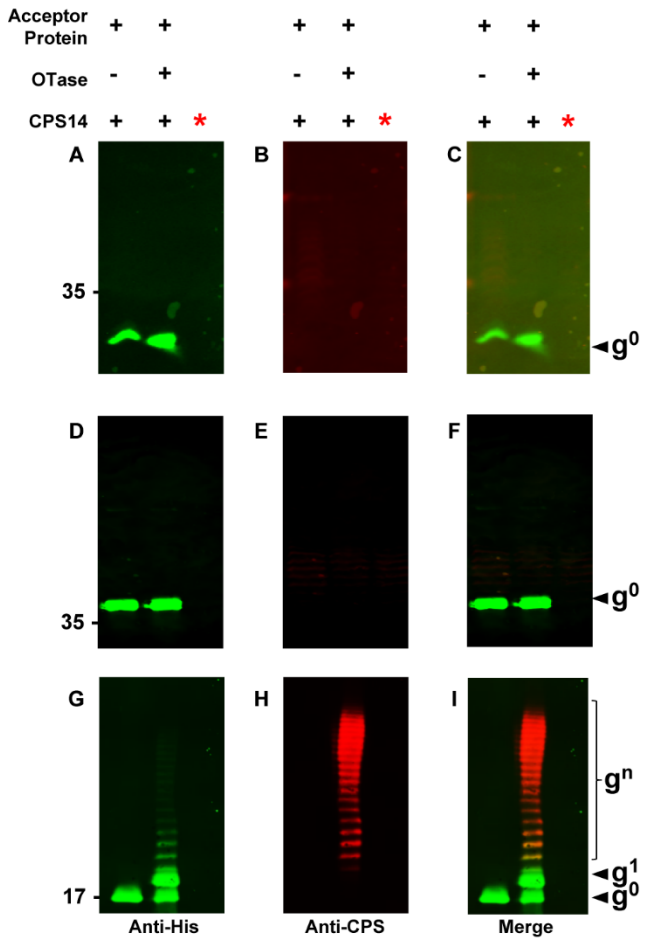


Figure 1. The oligosaccharyltransferase PglS can glycosylate the acceptor protein ComP with the pneumococcal CPS14 polysaccharide.

E. coli SDB1 cells co-expressing an acceptor protein (DsbA, AcrA, or ComP), an OTase (PglL, PglB, or PglS), and the CPS14 polysaccharide were analyzed for protein glycosylation via western blot analysis of the affinity purified acceptor proteins. *(A-C)* DsbA purified from SDB1 cells in the presence or absence of PglL. (*A)* Anti-His channel probing for hexa-histidine tagged DsbA. *(B)* Anti-glycan channel probing for CPS14. *(C)* Merged images for panels *A* and *B*. *(D-F)* AcrA purified from SDB1 cells in the presence or absence of PglB. *(D)*

Anti-His channel probing for hexa-histidine tagged AcrA. *(E)* Anti-glycan channel probing for CPS14. *(F)* Merged images for panels *D* and *E*. *(G-I)* ComP purified from SDB1 cells in the presence or absence of PglS. *(G)* Anti-His channel probing for hexa-histidine tagged ComP. *(H)* Anti-glycan channel probing for CPS14. *(I)* Merged images for panels *G* and *H*. The red asterisk indicates samples that were proteinase K treated for 1h at 55˚C.

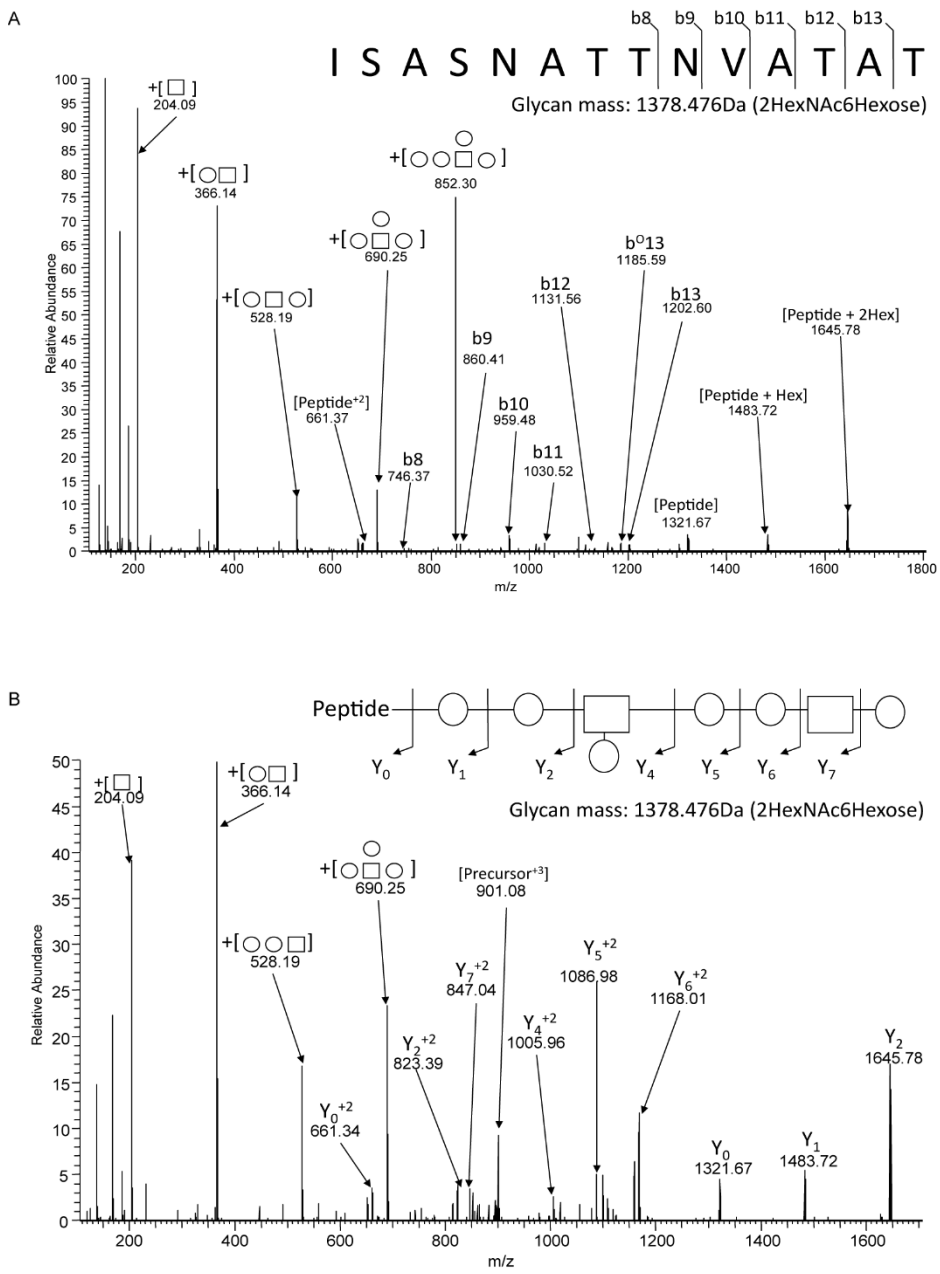


Figure 2. Higher energy collisional dissociation (HCD) fragmentation spectra of GluC digested CPS14-ComP bioconjugates.

GluC digested CPS14-ComP was subjected to HCD fragmentation enabling the confirmation of a single peptide attached to a glycan with the CPS14 repeating subunit. High collision energies *(A)* and low collision energies *(B)* regimes were undertaken to confirm the glycosylation of the peptide $_{81}$ISASNATTNVATAT$_{94}$ with a 1378.47 Da glycan corresponding to HexNAc$_2$Hexose$_6$.

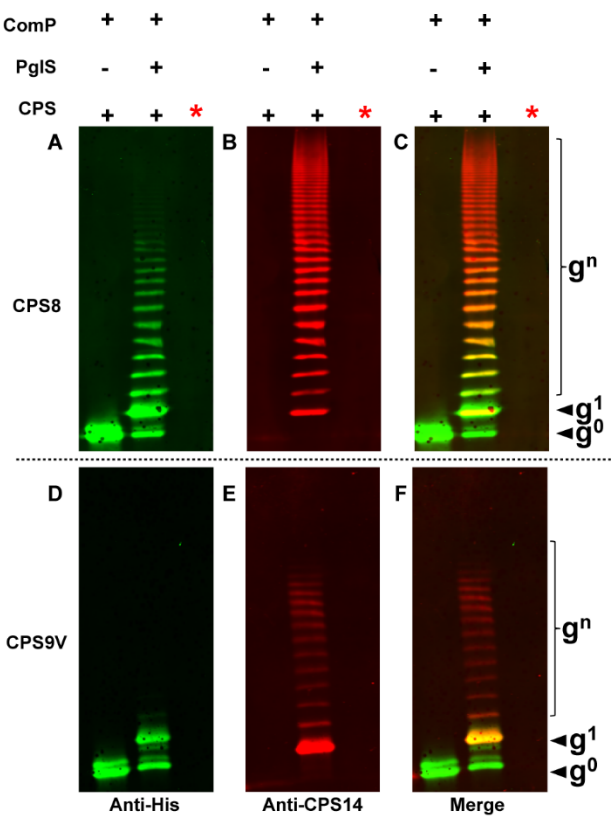


Figure 3. Western blot analysis of CPS8-ComP and CPS9V-ComP glycoproteins.

E. coli SDB1 cells were prepared co-expressing ComP, PglS, and either the pneumococcal CSP8 or CPS9V. Affinity purified glycosylated ComP from each strain was analyzed for protein glycosylation via western blot analysis. *(A-C)* Western blot analysis of CPS8-ComP bioconjugates compared against ComP alone *(A)* Anti-His channel probing for hexa-histidine tagged ComP purified from SDB1 expressing CPS8 in the presence or absence of PglS. *(B)* Anti-glycan channel probing for CPS8. *(C)* Merged images for panels *A* and *B*. *(D-F)* Western blot analysis of CPS9V-ComP bioconjugates compared against ComP alone *(D)* Anti-His channel probing for hexa-histidine tagged ComP purified from SDB1 expressing CPS9V in the presence or absence of PglS. *(E)* Anti-glycan channel probing for CPS9V. *(F)* Merged images for panels *D* and *E*. The red asterisk indicates samples that were proteinase K treated for 1h at 55˚C.

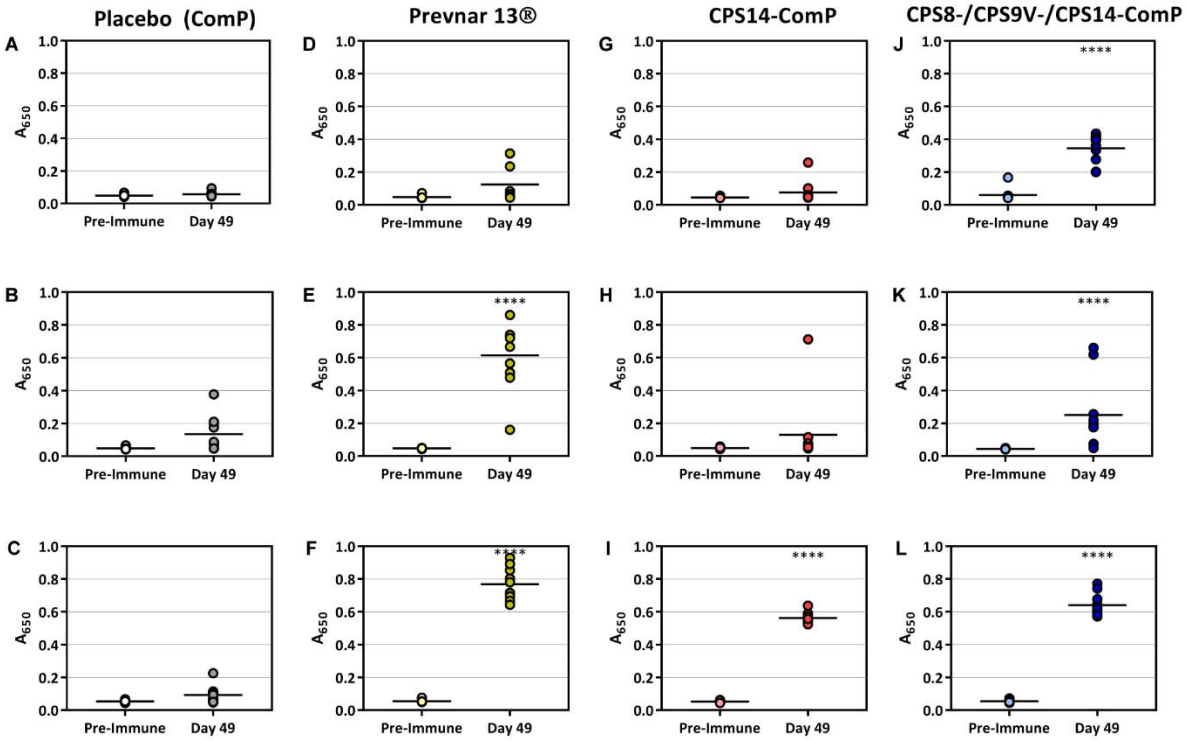


Figure 4. IgG responses of mice vaccinated with ComP, Prevnar 13®, a monovalent CPS14-ComP bioconjugate and a trivalent CPS8-/CPS9V-/CPS14-ComP biconjugate.

Groups of mice were vaccinated with ComP alone, Prevnar 13®, a monovalent CPS14-ComP bioconjugate vaccine, or a CPS8-/CPS9V-/CPS14-ComP biconjugate vaccine. Sera were collected on day 49 and analyzed for serotype specific IgG responses via ELISA compared against sera collected on day 0. *(A-C)* No IgG responses were detected in placebo vaccinated mice for serotypes 8 *(A)*, 9V *(B)*, or 14 *(C)*. *(D-F)* Prevnar 13® vaccinated mice did not have detectable IgG responses to serotype 8 *(D)*, but did have IgG responses specific to serotype 9V (E) and 14 (F). *(G-I)* Mice vaccinated with a CPS14-ComP bioconjugate vaccine did not have IgG responses to serotypes 8 *(G)* or 9V (H), but did have IgG responses to serotype 14 *(I)*. *(J-L)* Trivalent CPS8-/CPS9V-/CPS14-ComP bioconjugate vaccinated mice all had statistically significant IgG responses to serotypes 8 *(J)*, 9V *(K)*, and 14 *(L)*. Unpaired t-tests (Mann-Whitney) were performed to statistically analyze pre-immune sera from day 49 sera. *P* values for each case tested were **** p=0.0001. Each dot represents a single vaccinated mouse. Error bars indicate the standard deviation of the mean.

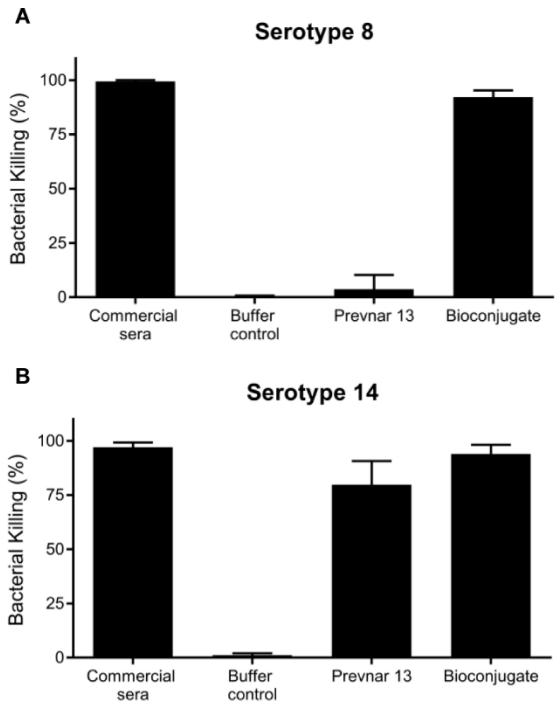


Figure 5. Bactericidal activity of sera from vaccinated mice against *S. pneumoniae* serotypes 8 and 14.

Opsonophagocytosis assays (OPA) of sera from mice vaccinated with either buffer control, Prevnar 13®, or bioconjugate vaccine against both *S. pneumoniae* serotypes 8 *(A)* and 14 *(B)*. Serotype-specific commercial rabbit anti-*S. pneumoniae* sera were used as positive controls. A 5% (v/v) sample serum and a bacterial MOI of 0.01 were added to fresh whole blood from naive mice to perform the assay. Viable bacterial counts were performed after 4 h of incubation. To determine bacterial killing, viable bacterial counts from tubes incubated with sample sera were compared to those incubated with control naive mouse sera. Results are expressed as percent bacterial killing for individual mice, with horizontal bars representing the standard deviation of the mean.

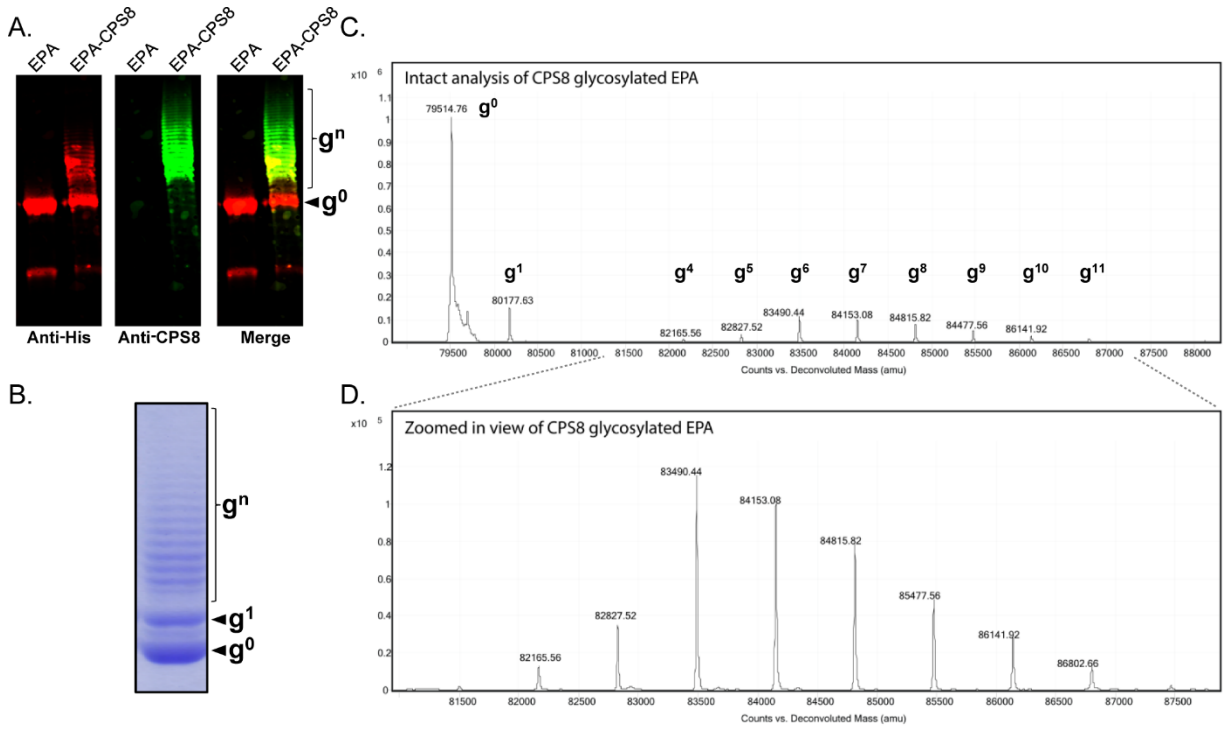


Figure 6. Analysis of EPA glycosylation with the CPS8 capsular polysaccharide.

Western blot analysis of EPA-CPS8 bioconjugates compared against EPA alone. *(A - Left panel)* Anti-His channel probing for hexa-histidine tagged EPA purified from SDB1 expressing CPS8 in the presence or absence of PglS. *(A – Middle panel)* Anti-glycan channel probing for CPS8. *(A – Right panel)* Merged images for left and middle panels. (*B)* EPA-CPS8 separated on a SDS polyacrylamide gel stained with Coomassie. *(C and D)* Intact protein mass spectrometry analysis showing the MS1 mass spectra for purified EPA-CPS8. The EPA fusion protein has a theoretical mass of 79,526.15 Daltons and can be observed as the peak at 79,514.76. The EPA fusion protein was also observed in multiple states of increasing mass corresponding to the CPS8 repeating subunit, which has a theoretical mass of 662 Daltons. Varying glycoforms of the EPA-CPS8 were observed and are denoted by "$g^{numeric}$" where "g" stands for glycoform and the "numeric" corresponds to the number of repeating CPS8 subunits. The EPA fusion protein was modified with up to 11 repeating subunits of the CPS8 glycan. Panel D provides a zoomed in view of the varying EPA-CPS8 glycoforms.

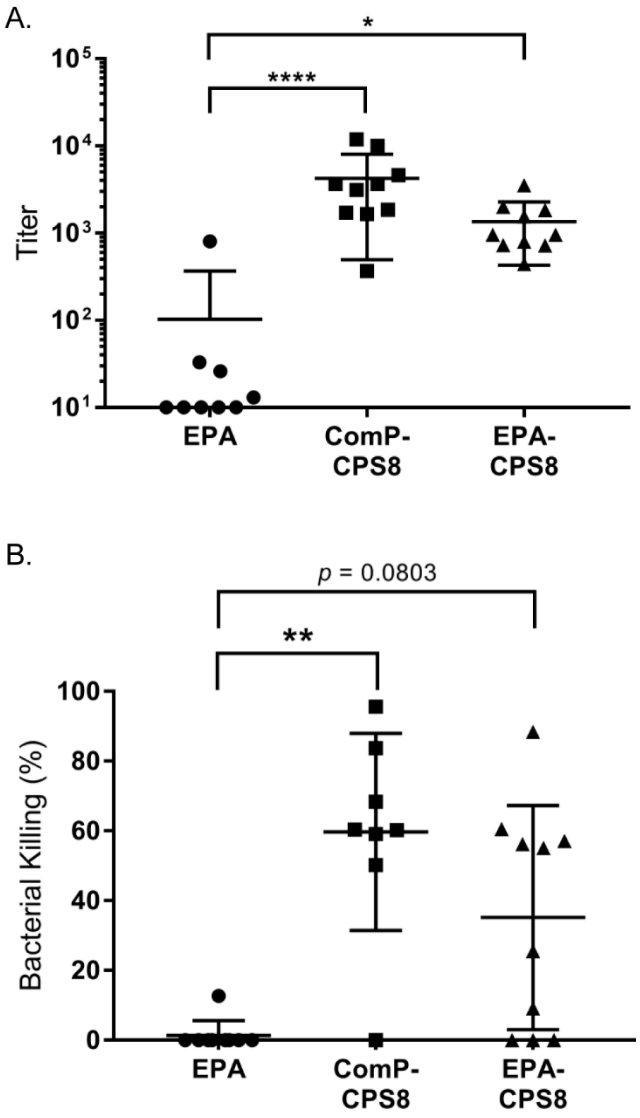


Figure 7. Analysis of immune responses to ComP-CPS8 and EPA-CPS8 bioconjugates in mice.

(A) Titers of CPS8 IgG antibodies in mice immunized with CPS8 bioconjugate vaccines. Mouse groups were as follows: EPA (n= 9, mice vaccinated with 5 µg of total protein), ComP-CPS8 (n=10, mice vaccinated with 5 µg total polysaccharide), and EPA-CPS8 (n=10, mice vaccinated with 100 ng of total polysaccharide). All mice were immunized with 100 µL of a vaccine diluted 1:1 with Imject Alum Adjuvant on days 1, 14, and 28. Sera were collected on day 4. For the titration, ELISA plates were coated with whole cell serotype 8 pneumococci

and incubated with 2-fold serial dilutions of sera. Titers for individual mice are shown, with horizontal bars representing the standard error of the mean. Statistically significant titers compared to the EPA placebo group are denoted with asterisk and were determined using Kruskal-Wallis one-way Anova. **, P=0.0223 and ****, P<0.0001. For analysis and representation purposes, negative titer values (<100) were given an arbitrary value of 10. *(B)* Opsonophagocytosis killing of *S. pneumoniae* serotype 8 by day 42 sera from mice immunized with ComP-CPS8 and EPA-CPS8 bioconjugate vaccines. The same mouse groups described for the IgG titers were employed for the OPA.A 40% (vol/vol) sample of serum and bacterial MOI of 0.01 were added to fresh whole blood from naïve mice to perform the assay. Results are expressed as percent bacterial killing for individual mice, with horizontal bars representing the standard error of the mean. Statistically significant killing compared to the EPA placebo group is denoted with asterisk and were determined using Kruskal-Wallis one-way Anova. **, P= 0.0015.

Supporting information

Supplementary material will become available with the article through the journal Web site upon publication.

Figure S1. Higher energy collisional dissociation (HCD) fragmentation spectra of GluC digested CPS14-ComP bioconjugates.

Figure S2. Point mutational analysis of ComP confirms serine 84 is the likely site of glycosylation.

Figure S3. Higher energy collisional dissociation (HCD) fragmentation spectra of GluC digested ComP glycosylated with the *C. jejuni* heptasaccharide (ComP-Glycan$_{Cj}$).

Figure S4. Higher energy collisional dissociation (HCD) fragmentation spectra of GluC digested ComP glycosylated with the *C. jejuni* heptasaccharide (ComP-Glycan$_{Cj}$).

Figure S5. Antibody responses towards monovalent CPS14-ComP bioconjugate vaccination.

Figure S6. A conserved and homologous serine is the likely site of glycosylation in ComP proteins from *A. baylyi* ADP1 and *A. soli* 110264.

Table S1. Bacterial strains and plasmids used in this study.

Table S2. Primers used in this study.