Université de Montréal

Rôle des composants de surface dans la pathogenèse de l'infection causée par *Streptococcus suis*

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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

Avril 2017

RÉSUMÉ

Les infections causées par les bactéries encapsulées d'importance en médecine humaine et vétérinaire, telle que *Streptococcus suis*, représentent un sérieux problème économique et en santé publique. La capsule polysaccharidique (CPS) et les protéines de surface sont des composants clés de la pathogenèse de *S. suis* en interagissant en première ligne avec les cellules de l'hôte durant la colonisation ainsi que lors de la dissémination de la bactérie pendant la phase systémique.

Streptococcus suis comporte 35 sérotypes basés sur l'antigénicité de la CPS. Or, l'impact de la structure et de la composition de la CPS, notamment la présence de l'acide sialique, sur ses propriétés fonctionnelles a été peu étudié. Pour leur part, les protéines de surface sont des cibles de choix pour des vaccins sous-unitaires, cependant plusieurs protéines caractérisées ont des rôles encore inconnus.

Les objectifs généraux de cette thèse sont, en premier lieu, de (i) investiguer l'impact de la structure et de la composition de la CPS dans la virulence de *S. suis*, (ii) d'étudier le rôle de l'acide sialique sur la synthèse et l'exportation de la CPS chez *S. suis,* et à titre de comparaison, chez le *Streptococcus* du groupe B (GBS) et (iii) d'étudier le rôle de certaines protéines de surface de *S. suis*, ayant à présent des fonctions inconnues, dans la pathogenèse de l'infection.

Les résultats présentés dans cette thèse démontrent premièrement que la CPS de *S. suis* est un important facteur antiphagocytaire indépendamment du sérotype testé. Des mutants déficients dans la synthèse de la CPS ont été produits chez *S. suis* sérotype 14 et ont permis de démontrer des propriétés antiphagocytaires similaires au sérotype 2. Ensuite, des souches de quatre différents sérotypes de *S. suis* ont été génétiquement modifiées afin d'exprimer une CPS d'un sérotype différent. Il y été ainsi démontré, sous un même bagage génétique, que la CPS de *S. suis* possédait effectivement des propriétés similaires entre les sérotypes testés. Afin

d'étudier l'effet de la composition de la CPS sur la virulence, et plus précisément le rôle de l'acide sialique contenu dans la CPS, nous avons caractérisé différents mutants des enzymes impliqués dans la sialylation de la CPS chez *S. suis* mais également chez GBS, à titre de modèle de comparaison. Nous avons confirmé avec plusieurs mutants le rôle critique de l'acide sialique dans la synthèse de la CPS chez *S. suis*, contrairement à GBS.

En étudiant le rôle des protéines de surface liant le facteur H humain (Fhb et Fhbp) dans la pathogenèse de *S. suis*, nous avons également démontré que la CPS participait aussi au recrutement du facteur H à la surface bactérienne. Les tests *in vitro* ont pu démontrer que le facteur H recruté à la surface de *S. suis* par Fhb et Fhbp favorisait l'adhésion aux cellules épithéliales et endothéliales. Finalement, il a été démontré que la protéine de surface 'Sao', malgré ses propriétés immunogéniques, ne représente pas un facteur de virulence critique pour *S. suis*.

Mots clés: *Streptococcus suis*, composants de surface, capsule polysaccharidique, acide sialique, facteur H, Fhb, Fhbp, Sao, virulence

ABSTRACT

Infections caused by encapsulated bacteria of importance in human and/or veterinary medicine, such as *Streptococcus suis*, represent a serious economic or public health problem. The capsular polysaccharide (CPS) and surface proteins are key components of *S. suis* pathogenesis by interacting with host cells during colonization and bacterial dissemination within the host during the systemic phase of the infection.

S. suis can be classified into 35 serotypes based on the CPS antigenicity. However, the impact of the CPS structure and composition, mainly the presence of sialic acid, on its functional properties has been little studied. In addition to the CPS, *S. suis* expresses several surface proteins. Yet, several characterized immunogenic proteins which have potential as sub-unit vaccine candidates, have unknown role in the pathogenesis of the infection caused by *S. suis*.

The general objectives of this thesis are (i) demystify the impact of CPS structure and composition in the virulence of *S. suis*, (ii) study the role of sialic acid on CPS synthesis and exportation in *S. suis*, and for comparison, in Group B *Streptococcus* (GBS) and (iii) study the role of some uncharacterized surface proteins in the pathogenesis of the infection caused by *S. suis*.

The results presented in this thesis demonstrated that the CPS expressed at the surface of *S. suis* is an important antiphagocytic factor independent of the tested serotype. Mutants deficient in CPS synthesis were produced in *S. suis* serotype 14 and results showed that this CPS has similar antiphagocytic properties that those of serotype 2 CPS. Furthermore, strains of four different *S. suis* serotypes were genetically engineered in order to express a CPS of a different serotype. It has thus been demonstrated, under the same genetic background, that *S. suis* CPS possesses similar properties among the serotypes evaluated, despite different compositions. In order to study the effect of CPS composition on virulence, and more

precisely that of capsular sialic acid, we have characterized different mutants of the enzymes involved in sialylation of the CPS in *S. suis*, but also in GBS, as a comparison model. We confirmed with several mutants the critical role of sialic acid in CPS synthesis in *S. suis*, in contrast to GBS.

By studying the role of factor H binding proteins (Fhb and Fhbp) in the pathogenesis of *S. suis*, we also demonstrated that the CPS is also implicated in the recruitment of factor H to the bacterial surface. The *in vitro* tests showed that factor H recruited at the surface of *S. suis* (partially by Fhb and Fhbp) promotes adhesion to epithelial and endothelial cells. Finally, it was demonstrated that the surface protein 'Sao', despite its immunogenic properties, is not a critical virulence factor for *S. suis*.

Keywords: *Streptococcus suis*, surface components, capsular polysaccharide, sialic acid, factor H, Fhb, Fhbp, Sao, virulence

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LISTE DES ABRÉVIATIONS

AMP	adénosine monophosphate
AMPc	AMP cyclique
BmDCs	« bone marrow-Derived Cells »
Ccpa	« catabolite control protein A »
CCR	« carbon catabolite repression»
CMP	cytidine-5′-monophosphate
CPS	capsule polysaccharidique
CRE	« catabolite response element »
CTP	cytosine triphosphate
EF	« extracellular factor »
Gal	galactose
GBS	streptocoque du groupe B
Glc	glucose
GlcNAc	*N*-acétylglucosamine
hBMEC	cellules endothéliales microvasculaires du cerveau humaine
Ig	immunoglobuline
IL	interleukine
LOS	lipooligosaccharide
LPS	lipopolysaccharide
ManNAc	*N*-acétylmannosamine
MCP-1	« monocyte chemotactic protein 1 »
MLST	« multilocus sequence typing »
MMP-9	métalloprotéinase matricielle 9
MRP	« muraminidase-release protein »
Neu5Ac	acide *N*-acétylneuraminique
Neu5Ac-CMP	« cytidine-5′-monophospho-*N*-acetylneuraminic acid »
PAMP	« pathogen associated molecular patterns »
pBMEC	cellule endothéliale microvasculaire du cerveau porcine

PCR	réactions en chaînes par polymérase
PGE2	prostaglandine E2
PRR	« pattern recognition receptors »
PTS	« phosphotransferase system »
Rha	rhamnose
Sao	« surface antigen one »
SCR	« short consensus repeat »
Siglecs	« sialic acid-binding immunoglobulin-like lectins »
SLY	suilysine
SNP	« single nucleotide polymorphism »
TCS	« two-components system »
TEM	« transmission electron microscopy»
TNF	« tumor necrosis factor »
UDP	uridine diphosphate
Und-P	« undecaprenyl-phosphate »

REMERCIEMENTS

Je tiens à remercier toutes les personnes qui m'ont aidé, soutenu et encouragé durant ce long périple qu'est un projet de doctorat, et plus particulièrement;

À ma directrice de doctorat, Dre Mariela Segura, qui m'a accueilli dans son laboratoire, de la maîtrise jusqu'au doctorat. Je vous remercie pour vos conseils, les opportunités de stages internationaux, mais également de m'avoir inculqué votre rigueur scientifique,

À mes codirecteurs, Dr Marcelo Gottschalk et Dr Nahuel Fittipaldi, qui ont été des personnes déterminantes dans la réussite de mon projet de doctorat, merci pour votre disponibilité, votre appui et vos conseils,

À Sonia, merci pour ces discussions (et les poutines) sur tout et sur rien, mais surtout pour ta disponibilité, ton écoute et ta bonne humeur quotidienne

À mes parents et ma famille qui m'ont apporté leur soutien indéfectible à travers les moments difficiles,

À tous mes amis et collègues présents et passés; Damian, Gaël, Annabelle, Marie-Pier, Claude, Jean-Philippe, Guillaume, Audrey, Léa, Claudia, Paul, Cynthia, pour leur camaraderie, leurs aides et leurs collaborations,

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. *S. suis* est l'un des pathogènes les plus problématiques chez les porcelets, pouvant causer septicémie, la mort subite, méningite, arthrite et endocardite. Chez l'humain, *S. suis* est responsable d'infection systémique, du syndrome du choc toxique ainsi que de septicémie, d'endocardite et également de méningite. En effet, la forme d'infection la plus commune et la plus frappante causée par *S. suis* est la méningite.

Il est proposé que *S. suis* colonise d'abord les voies respiratoires supérieures chez le porc afin de gagner la circulation sanguine où il peut se multiplier et se disséminer. La capsule polysaccharidique (CPS) et les protéines de surface sont des composants clés de la pathogenèse de *S. suis* en interagissant directement avec les cellules de l'hôte durant la colonisation et la dissémination. Les propriétés antiphagocytaires font de la CPS de *S. suis* l'un des facteurs de virulence le plus important, notamment pour son rôle dans l'évasion des défenses immunitaires de l'hôte. La CPS de *S. suis* est également le composant de surface à la base du sérotypage. En effet, 35 sérotypes basés sur l'antigénicité de la CPS ont été décrits chez *S. suis*. Le sérotype 2 est le type capsulaire le plus isolé autant chez le porc que chez l'humain et, quant à lui, le sérotype 14 est considéré comme un sérotype émergeant en Amérique du Nord avec de récents cas humains déclarés au Canada. D'autres sérotypes sont fréquemment associées à des infections à *S. suis* chez le porc, dont les sérotypes 1, 1/2, 3 et 9.

Une particularité de la CPS de *S. suis* est la présence d'acide sialique chez certains sérotypes. Étonnamment, chez les bactéries à Gram positif, seul *S. suis* et le streptocoque du groupe B (GBS) incorporent de l'acide sialique dans leurs CPSs. Il est proposé que la CPS composée d'acide sialique pourrait augmenter la virulence de la bactérie en inhibant l'activation de la voie alternative du complément, altérer les voies de signalisation impliquées dans la phagocytose et/ou réduire la capacité bactéricide des neutrophiles. Malgré une composition en sucres similaire, une différence importante est la nature du lien de l'acide sialique avec le galactose adjacent au sein de la CPS entre *S. suis* (α2,6-Gal) et GBS (α2,3-Gal). La variation dans la structure conformationnelle exercée par l'acide sialique pourrait avoir un impact inattendu sur les réponses immunitaires de l'hôte face à ces deux pathogènes.

Le caractère peu immunogénique de la CPS et son rôle dans l'évasion immunitaire représentent un sérieux casse-tête dans le développement des vaccins contre *S. suis*. En effet, en plus de masquer partiellement des protéines immunogéniques, la CPS induit de très faibles niveaux d'anticorps lors d'une infection. Cependant, l'impact de la structure et de la composition (notamment de l'acide sialique) de la CPS sur la virulence de *S. suis* et la modulation de la réponse immunitaire ont été peu étudiés et pourraient fournir des informations clés sur les propriétés immunogéniques de la CPS.

Pour leur part, les protéines de surface sont des cibles de choix pour des vaccins sous-unitaires, cependant plusieurs protéines immunogéniques caractérisées ont des rôles encore inconnus dans la pathogenèse de l'infection causée par *S. suis*. La majorité des protéines de surface décrites à ce jour ont des rôles dans l'adhésion du pathogène aux composants de l'hôte et sont impliquées dans la colonisation et/ou la dissémination. Certaines protéines de surface non caractérisées, dont Sao (pour 'surface antigen one'), pourraient ainsi avoir des rôles critiques dans différentes étapes de la pathogenèse de l'infection. De plus, récemment, deux protéines de surface, Fhb et Fhbp (pour 'factor H-binding proteins'), ont été caractérisées pour leur capacité à lier le facteur H (un régulateur majeur de la voie alternative du complément). Il a été démontré que plusieurs pathogènes recrutent le facteur H à leur surface afin de se protéger contre le complément. De plus, le facteur H recruté à la surface de certains pathogènes contribue à l'adhésion de la bactérie à la matrice extracellulaire et à l'invasion des cellules de l'hôte. Néanmoins, le rôle de ces deux protéines liant le facteur H dans la pathogenèse de *S. suis* demeure inconnu.

L'**hypothèse générale** de cette thèse est que les composants de surface de *S. suis*, tels la CPS et les protéines de surface influencent directement la pathogenèse de l'infection causée par *S. suis*. Plus précisément, les hypothèses spécifiques sont que (i) La structure et la sialylation de la CPS de *S. suis* influencent ses propriétés antiphagocytaires et/ou immunomodulatrices, et (ii), les protéines de surface de *S. suis* représentent *de facto* des facteurs de virulence importants.

Les **objectifs généraux** de cette thèse sont, en premier lieu, d'investiguer l'impact de la structure et de la composition de la CPS dans les interactions hôte-pathogène et la virulence de *S. suis* et dans un second temps, étudier le rôle de certaines protéines de surface de *S. suis* ayant à présent des fonctions inconnues dans la pathogenèse de l'infection.

Dans l'ordre abordé dans cette thèse, les objectifs spécifiques sont :

I- Étudier l'impact de la structure de la CPS des différents sérotypes de *S. suis* dans les interactions hôte-pathogène. Cet objectif s'intéressera plus particulièrement à étudier les propriétés antiphagocytaires de la CPS d'importants sérotypes de *S. suis* et d'étudier l'impact de la CPS de ces sérotypes sur la virulence dans un modèle in vivo murin. La mutagenèse ciblée a été utilisée pour muter des gènes du locus capsulaire afin d'inhiber la synthèse de la CPS chez le sérotype 14 et, dans un second temps, afin de créer des mutants isogéniques exprimants des CPSs antigéniquement différentes chez les sérotypes 1/2, 1, 2 et 14.

II- Étudier le rôle de l'acide sialique sur la synthèse et l'exportation de la CPS chez *S. suis,* et à titre de comparaison, chez GBS. Cet objectif s'intéressera à l'impact de l'acide sialique chez les bactéries à Gram positif sur la synthèse et les propriétés immunogéniques de la CPS. De plus, une approche de mutagenèse afin de créer des souches présentant différents épitopes d'acide sialique a été développée pour la réalisation de cet objectif en substituant les gènes codant pour les sialyltransférases chez *S. suis* (α2,6) et GBS (α2,3).

III- Caractériser le rôle de certaines protéines de surface ancrées à la paroi dans la pathogenèse de l'infection causée par *S. suis.* Cet objectif jettera un regard sur le rôle de la protéine de surface Sao et des protéines de surface liant le facteur H humain, dans la virulence et dans la pathogenèse de l'infection causée par *S. suis*.

À travers ce document nous démontrons que la CPS de *S. suis* un est facteur antiphagocytaire critique indépendamment du sérotype et est essentiel pour la virulence de ce pathogène. De plus, nous avons confirmé par deux approches différentes, le rôle critique de l'acide sialique dans la synthèse de la CPS chez *S. suis*. La comparaison du rôle de l'acide

sialique entre *S. suis* et GBS a permis de démontrer que cette dernière particularité est propre à *S. suis*.

Dans un second temps, nous avons démontré que, malgré ses propriétés immunologiques, la protéine de surface Sao n'est pas un facteur critique pour la virulence du pathogène *S. suis*. De plus, nous avons démontré que certaines protéines de surface liant le facteur H contribuent à l'adhésion aux cellules de l'hôte et conséquemment, contribue à la colonisation de *S. suis*.

Ces travaux ont contribué à l'avancement des connaissances fondamentales sur le rôle des composants de surface dans la pathogenèse de l'infection causée par *S. suis* et ont également permis de créer des outils (mutants) qui pourront être utilisés pour étudier plus en profondeur les facteurs bactériens contribuant à la virulence de *S. suis*.

II. REVUE DE LITTÉRATURE

1. *Streptococcus suis*

Streptococcus suis est une bactérie à Gram positif encapsulée dont l'habitat naturel principal est les voies respiratoires supérieures du porc, plus particulièrement les amygdales et les cavités nasales[1, 2]. On retrouve néanmoins également ce pathogène au niveau de la flore digestive et de la flore vaginale du porc[3]. Ce pathogène zoonotique cause des infections chez le porc et également chez l'humain. D'ailleurs, les souches isolées de ces deux hôtes partagent fréquemment des caractéristiques phénotypiques et génotypiques similaires[4].

Les principaux signes cliniques causés par l'infection chez l'homme et le porc sont la méningite et la septicémie[1]. Les infections à *S. suis* chez l'homme sont majoritairement reportées dans les pays Asiatiques, qui comptent pour plus de 90% des cas d'infections humaines. Dans certains pays Asiatiques, notamment au Vietnam, les infections à *S. suis* chez l'homme sont surtout reliés à la consommation de viandes crues infectées. Néanmoins, on recense également plusieurs cas d'infection dans les pays Asiatiques auprès des travailleurs qui entrent en contact avec des porcs infectés. Les coutumes et l'hygiène sont les principales causes d'infection à *S. suis* dans les pays Asiatiques[3].

En effet, l'intérêt pour ce pathogène a connu une hausse importante suite à l'épidémie de *S. suis* dans la province de Sichuan en Chine en 2005, comme le démontre la **Figure 1**[5, 6]. Il s'agissait de la deuxième épidémie de *S. suis*, la première eu lieu en 1998, également en Chine[7]. Le nombre d'articles scientifiques publiés concernant *S. suis* à significativement augmenté depuis le début des années 2000, et particulièrement après l'épidémie de 2005. C'est d'ailleurs la Chine (45%) qui publie le plus grand nombre d'articles scientifiques sur *S. suis* (pour la période de 2008 à 2012), suivi du Canada (11%) et de l'Allemagne (6%). Durant l'éclosion de *S. suis* de 2005, plus de 215 cas humains ont été déclarés dans la seule province de Sichuan, dont 39 sont décédés de l'infection. Le taux de mortalité élevé a été une des caractéristiques particulièrement inquiétante de cette éclosion, dont l'hypothèse (confirmée) de l'origine est l'émergence d'une souche hautement virulente[5].

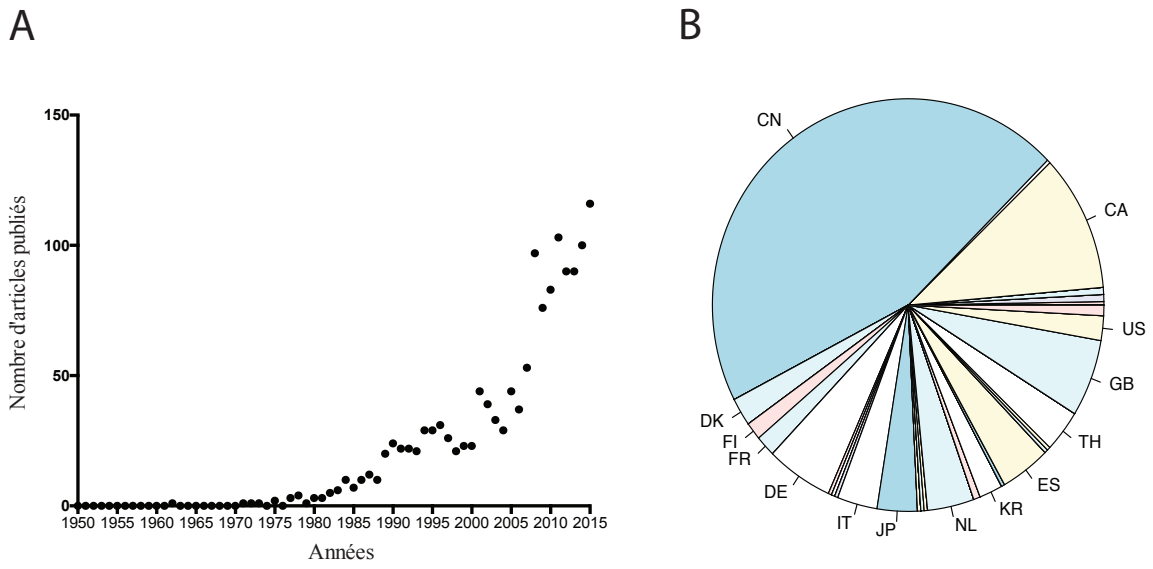


Figure 1. Graphique représentant le nombre d'articles sur *S. suis* publiés en fonction des années (A) et des pays (B). CN; Chine, CA; Canada, US; États-Unis, GB; Grande-Bretagne, TH; Thaïlande, ES; Espagne, KR; Corée, NL; Pays-Bas, JP; Japon, IT; Italie, DE; Allemagne, FR; France, FI; Finlande, DK; Danemark.

En Amérique du Nord, les cas humains d'infections à *S. suis* sont très rares, soit 0.5% de l'ensemble des cas humains répertoriés et sont considérées comme des maladies professionnelles. Par contre, curieusement, c'est en Amérique de Nord qu'on recense le plus grand nombre de cas d'infection chez le porc, soit plus de 65% des cas déclarés dans le monde. À titre d'exemple, les pays asiatiques ne représentent que 14% des cas d'infections déclarées chez le porc. Une cause probable de ce phénomène serait le faible de taux de diagnostic ou de déclaration d'infection à *S. suis* chez le porc dans certains pays asiatiques tels que la Thaïlande et le Vietnam, en comparaison avec l'Amérique du Nord[3].

1.2. Typage

Il existe deux méthodes de typages chez *S. suis*. La première est le typage sérologique, qui différencie les souches de *S. suis* selon l'antigénicité de la CPS (section 1.2.1). Le typage

sérologique se fait de routine par le test de coagglutination[8]. Néanmoins, la présence de certains gènes capsulaires permet de prédire l'antigénicité potentiel de la CPS et des tests par réactions en chaîne par polymérase (PCR) multiplexe (PCR multiplexe) ont été développés afin de déterminer le sérotype. Ce type de typage regroupe *S. suis* selon le type de CPS potentiellement exprimée et encodée par les différents gènes associés à la synthèse de la CPS[9, 10]. Le sérotypage par PCR multiplexe possède donc un avantage (ou désavantage), soit être capable d'associer la souche à un type capsulaire malgré l'absence de l'expression de la CPS (section 1.2.2). La seconde méthode de typage est le typage allélique qui regroupe *S. suis* en fonction de son bagage génétique (section 1.2.3). On fait alors référence au type allélique ou « sequence type » (ST)[11].

1.2.1. Tests sérologiques et sérotypes existants

La majorité des laboratoires de diagnostic sur *S. suis* utilisent le test sérologique de coagglutination afin de déterminer le sérotype des souches de *S. suis*. Le test de coagglutination utilise la protéine A exprimée à la surface de *Staphylococcus aureus* afin de lier la portion Fc d'une immunoglobuline (Ig) de classe G (IgG), laissant la portion Fab de l'IgG libre de lier son antigène[12]. Ainsi, un sérum de lapin immunisé avec la souche de référence d'un sérotype donné, sera capable d'agglutiner les cellules de *Staphylococcus aureus* en présence de leur antigène, soit la CPS appartenant au même sérotype de *S. suis*.

Dans les années 1980 à 1990, 35 sérotypes différents de *S. suis* ont été ainsi caractérisés basé sur l'antigénicité de la CPS, soit les sérotypes 1 à 34 et le sérotype 1/2[13]. Cette méthode largement utilisée est relativement fiable pour la plupart des souches testées. Par contre, le sérotypage comporte certaines limites; dont (i) la présence de réactions croisées [3, 14], et (ii) l'existence de souches non-typables ou autoagglutinantes[15, 16].

(i) Les réactions croisées, qui sont la reconnaissance d'au moins deux antigènes différents par le même sérum, existent entre plusieurs sérotypes. Par exemple, le sérum contre le sérotype 1 reconnaît aussi les antigènes du sérotype 1/2 et du sérotype 14. À l'opposé, le sérum contre le

sérotype 14 reconnaît également le sérotype 1. Le sérum contre le sérotype 6 donne une réaction croisée avec le sérotype 15, et finalement le sérum du sérotype 2 réagit aussi avec les antigènes du sérotype 1/2 et du sérotype 22[3]. À l'écriture de cette thèse, les causes précises de ces réactions croisées sont encore inconnues. Néanmoins, certaines hypothèses dues à la similarité des structures seront discutées à la section 2.

(ii) La seconde limite technique du test de coagglutination est l'existence de souches non-typables ou autoagglutinantes. Les souches non-typables sont des souches qui ne réagissent avec aucun antisérum. Pour leur part, les souches autoagglutinantes démontrent de l'agglutination dans la solution contrôle, rendant le sérotypage impossible.

Au moment d'amorcer cette thèse, très peu d'information était disponible sur ces souches non-typables et autoagglutinantes. Une hypothèse est que les souches non-typables et autoagglutinantes représentent des souches ayant perdu la capacité de synthétiser la CPS et ont un phénotype non encapsulé. Une autre hypothèse est que plusieurs de ces souches appartiennent à de nouveaux sérotypes non caractérisés à ce jour.

Récemment avec la disponibilité des technologies d'analyses génétiques moléculaires, il a été proposé que les sérotypes 20, 22, 26, 32, 33 et 34 appartiennent à une autre espèce que *S. suis*. Dans les cas des sérotypes 20, 22, 26 et 33, les comparaisons des séquences des gènes hautement conservés *sodA* et *recN* ont permis de confirmer que ces sérotypes appartenaient à une autre espèce que *S. suis*[17]. Dans le cas des sérotypes 32 et 34, l'analyse comparative des séquences des gènes hautement conservés *cpn60* et de l'ARN 16S a permis de reclassifier ces sérotypes comme étant *S. orisratti*[18]. Après ces reclassifications, *S. suis* comporterait donc 29 différents sérotypes caractérisés[13].

1.2.2. Typage moléculaire (« Multiplex PCR »)

Afin de contourner les limites décrites ci-haut concernant les tests sérologiques, des tests moléculaires basés sur l'amplification de gènes spécifiques au sérotype se sont

développés. Il existe présentement trois types de PCR multiplexe permettant d'identifier l'ensemble des sérotypes de *S. suis* par biologie moléculaire. La PCR mutiplexe est une variante de la PCR standard qui permet l'utilisation de plusieurs amorces amplifiant des produits de différentes tailles. Il existe également plusieurs autres tests se limitant à la détection des sérotypes les plus communs. Il est à noter que certaines souches non-sérotypables demeurent non-typables par ces techniques d'identification moléculaires.

Le premier test a été développé par une équipe japonaise (Okura & al.)[19] et s'effectue en deux étapes dépendantes. La première est le regroupement des souches en sept groupes différents selon l'amplification de gènes codant pour la CPS communs à plusieurs sérotypes. La seconde étape est la détermination du sérotype par l'amplification du gène de la polymérase, un gène sérotype-spécifique. Malgré la présence d'une réaction contrôle amplifiant le gène de l'ARNr 16S de *S. suis*, cette technique conduit, cependant, à la mauvaise identification des sérotypes 20, 22, 26, 32, 33 et 34 comme appartenant à l'espèce *S. suis*[19].

Le deuxième test a été développé par une équipe chinoise (Liu & al.)[20] et utilise quatre ensembles d'amorces indépendantes afin d'identifier 33 sérotypes de *S. suis*. Chaque ensemble d'amorces doit être successivement utilisé à moins d'un résultat positif, afin de déterminer le sérotype. Pour sa part, ce test utilise l'amplification spécifique du gène hautement conservé *thrA* comme contrôle positif à l'appartenance à l'espèce *S. suis* et ne considère pas les sérotypes 32 et 34, reclassifiés comme *S. orisratti*[20].

Un dernier test, développé récemment par une équipe japonaise (Kerdsin & al.)[10], fonctionne également avec quatre ensembles d'amorces permettant d'amplifier différents gènes codant pour la synthèse de la CPS. Un avantage de ce test est qu'il permet l'identification des 29 'vrais' sérotypes de *S. suis* et ne considère plus les sérotypes 20, 22, 26, 32, 33 et 34, qui appartiennent à une autre espèce que *S. suis*[10].

1.2.3. Typage allélique MLST « Multiple Locus Sequence Type »

Le typage moléculaire allélique ou « mulitple locus sequence type » (MLST) est une technique de caractérisation de souches au sein d'une espèce bactérienne en se basant sur des séquences internes de sept gènes hautement conservés. Le typage allélique chez *S. suis* se base sur des variations dans des gènes conservés *aroA, cpn60, dpr, gki, mutS, recA* et *thrA*. Chaque séquence interne des gènes, appelée allèles, donneront un numéro d'allèle afin de créer un profil composé des sept allèles des gènes cibles. Ce profil est ensuite associé à un type allélique ou « sequence type » (ST) précis[11].

Cette technique ne considère pas l'ensemble des polymorphismes nucléotidiques (SNP) de la séquence, mais cible seulement quelques variations précises au sein de la séquence. Cette méthode a gagné beaucoup en popularité dans les dernières années avec la disponibilité des technologies de séquençage ainsi que de la banque d'allèle et de profils disponibles facilement en ligne (*www.pubmlst.org*)[11].

Au moment d'écrire cette thèse, 833 différents types alléliques de *S. suis* ont été caractérisés. Cependant, plusieurs types alléliques sont étroitement reliés, suggérant un ancêtre commun pour plusieurs des différents STs. Il semble y avoir une association entre la virulence d'une souche et le ST. Le type allélique ST1 est le complexe le plus fortement associé aux cas d'infections sévères caractérisées par une septicémie, une méningite ou de l'arthrite[7]. De plus, dans les deux épidémies de *S. suis* qui ont eu lieu en 1998 et en 2005, les souches responsables étaient, dans les deux cas, du type allélique ST7[21]. Certains STs sont associés avec la présence de gènes codant pour certains marqueurs de virulence comme la suilysine (*sly*), la « muraminidase-released protein » (*mrp*) et le facteur extracellulaire (*ef*). La plupart des types alléliques ST1 et ST7 possèdent tous les marqueurs de virulences *sly, mrp* et *ef* alors que les types alléliques ST25 et ST28, souvent associés à des souches moins virulentes ou peu virulentes, sont pour la plupart *sly* et *ef* négatifs[22]. Curieusement, on peut retrouver le même sérotype dans des STs qui sont extrêmement différents, ce qui suggère que les gènes de la CPS peuvent être transférés horizontalement.

1.3. Épidémiologie

1.3.1. Distribution des sérotypes

La distribution des différents sérotypes de *S. suis* isolés de porcs infectés varie considérablement en fonction de la localisation (pays/continent). Néanmoins, le sérotype 2 demeure le sérotype le plus isolé dans le cas d'infection à *S. suis* chez le porc. En Amérique du Nord, on retrouve particulièrement les sérotypes 2, 1/2 et 3 dans les cas de porcs infectés. C'est d'ailleurs en Amérique du Nord qu'il y a le plus grand nombre de cas répertoriés d'infection à *S. suis* chez le porc. Curieusement, au Canada le sérotype le plus isolé est le sérotype 2, alors chez les États-Unis on isole davantage le sérotype 3. Néanmoins, ensemble, les sérotypes 2 et 3 représentent les deux sérotypes les plus présents en Amérique du Nord.

En Europe, ce sont particulièrement les sérotypes 2, 3 et 9 qui sont responsables d'infection à *S. suis* chez le porc. En Asie, où les infections à *S. suis* peuvent être considérées comme endémiques, ce sont les sérotypes 2, 3, 4, 7 et 8 qui sont les plus isolés dans le cas d'infection chez le porc. Dans le cas de la Chine, on isole principalement le sérotype 2, alors qu'en Corée il s'agit davantage du sérotype 3. Étonnamment, la Chine est le troisième pays avec le plus de cas répertoriés chez le porc (639), derrière l'Espagne (666) et le Canada (3065), sur une même période[3].

En contraste avec les infections à *S. suis* chez le porc, les infections chez l'humain sont presque exclusivement causées par le sérotype 2 de *S. suis*. Le sérotype 14 vient ensuite loin derrière avec 2% des cas répertoriés chez l'humain. C'est en Asie qu'on retrouve le plus grand nombre de cas d'infection à *S. suis* chez l'humain avec plus de 90% des cas répertoriés. La proximité des élevages, la consommation de viande crue et l'absence de règlementation sont des causes qui expliquent en partie le nombre de cas élevé répertorié ce continent. En Asie, les pays où l'on répertorie le plus grand nombre d'infections chez l'humain sont le Vietnam (574), la Thaïlande (553) et la Chine (245), pour la même période (2002-2013). Étonnamment, l'Amérique du Nord qui compte le plus grand nombre de cas d'infection chez le porc, est l'une des régions que l'on recense le moins de cas d'infections chez l'humain à *S. suis* avec seulement 8 cas déclarés (0.5% des cas répertoriés)[3].

1.3.2. Distribution des STs

Il existe une certaine distribution géographique des types alléliques dans le monde. Le ST1 est surtout retrouvé en Asie, en Europe et en Argentine, alors que les ST25 et ST28 sont davantage retrouvés en Amérique du Nord. Le type allélique ST28 est également retrouvé fréquemment au Japon avec le type allélique ST1. Le type allélique ST1 est le principal type allélique responsable des infections à *S. suis* autant chez l'humain que chez le porc dans les pays Asiatiques et Européens, alors qu'en Amérique du Nord les infections à *S. suis* chez le porc sont principalement causées par les ST25 et ST28. Finalement, un autre type allélique isolé lors des épidémies de *S. suis* en Chine, le ST7, a été responsable de plusieurs cas d'infections chez le porc et l'humain durant ces épidémies[21]. Ce type allélique est d'ailleurs endémique à la Chine[3, 22].

1.4. Pathogenèse de l'infection et réponse inflammatoire

S. suis est une bactérie ayant la capacité de causer une maladie et est considérée comme un pathogène[1]. Les infections causées par *S. suis* sont surtout retrouvées chez les jeunes porcelets en bas de 16 semaines[23]. Il a été observé que la majorité des cas d'infections se produisent durant le sevrage entre la troisième et la douzième semaine de vie des porcelets[24]. La contamination des porcelets avec *S. suis* peut se faire par transfert vertical à partir de la mère colonisée, mais également à partir des autres porcs qui entrent en contact avec ceux-ci[3]. Il est à noter que la majorité des porcs en élevage sont porteurs de la bactérie *S. suis*[2]. Certains animaux ne présenteront par contre jamais de signes cliniques de l'infection, et sont considérés comme des porteurs sains. Chez les porteurs sains, *S. suis* colonise les voies respiratoires supérieures, notamment les amygdales, et peut ainsi se transmettre par transfert horizontal aux autres animaux. Dans le cadre de cette thèse, nous discuterons davantage de la pathogenèse de l'infection initiée par la colonisation des voies respiratoires supérieures du porc par *S. suis*. En revanche, chez l'humain, la principale cause de contamination est via des lésions cutanées en manipulant des porcs malades ou via la consommation ou la manipulation de viande contaminée[3, 25].

Globalement, les étapes de la pathogenèse de l'infection causée par les streptocoques pathogènes se traduisent en trois grandes étapes, soit (i) la colonisation ou l'association avec les cellules épithéliales de l'hôte, (ii) multiplication et évasion des défenses immunitaires de l'hôte, et puis (iii) les dommages à l'hôte. Dans un cycle infectieux, il y a une dernière étape soit (iv) la transmission à un autre hôte[26].

Lors de la première étape de l'infection, soit (i) la colonisation des cellules épithéliales de l'hôte, *S. suis* a recours à plusieurs adhésines qui lui permettent d'interagir avec les composants membranaires des cellules épithéliales des voies respiratoires supérieures du porc. Généralement, l'adhésion de la bactérie aux cellules de l'hôte s'effectue via des interactions hydrophobiques, liens cationiques et via des liaisons récepteurs-ligands[27]. *S. suis* peut également utiliser la matrice extracellulaire présente à la surface des cellules de l'hôte afin de favoriser l'adhésion (indirecte) aux cellules. Parmi les composants de la matrice extracellulaire souvent utilisés par *S. suis*, on y retrouve la fibronectine, la laminine et le collagène[27]. Suite à l'association avec les cellules de l'hôte, et par des mécanismes encore peu décrits, il est proposé que *S. suis* traverse ensuite la barrière épithéliale pour gagner la circulation sanguine[28].

Une fois la circulation sanguine rejointe, *S. suis* peut exprimer plusieurs composants de surface, dont la CPS, permettant l'évasion des défenses de l'hôte. En effet, pour survivre dans le sang, *S. suis* est capable de diminuer la capacité bactéricide des neutrophiles, éviter la phagocytose par les cellules phagocytaires professionnelles (dont les macrophages) et même dégrader certaines immunoglobulines[29, 30, 31, 32, 33, 34].

À ce niveau, les souches virulentes de *S. suis* peuvent alors causer la septicémie, qui se caractérise par une infection aiguë du sang. Les bactéries circulant dans le sang peuvent également gagner d'autres sites et organes et causer d'autres maladies telles que l'endocardite et l'arthrite[1, 6].

Il est ensuite proposé que *S. suis*, via la circulation sanguine, rejoint la barrière hématoencéphalique. Pour ce faire, une hypothèse est la théorie du cheval de Troie modifiée,

selon laquelle *S. suis* gagnerait le système nerveux central (SNC) en étant associé à la surface externe de monocytes grâce à des adhésines, en plus de voyager libre en circulation[35]. Au niveau de la barrière hématoencéphalique, certaines toxines et/ou enzymes seraient alors impliquées dans la diminution de l'étanchéité de la barrière et permettrait ainsi à *S. suis* de gagner le SNC et causer la méningite. La méningite est un des signes cliniques le plus caractéristique d'une infection à *S. suis*. Il est d'ailleurs proposé que la suilysine, une hémotoxine de *S. suis*, est impliquée dans la perméabilisation de la barrière hématoencéphalique[36]. Les caractéristiques de la suilysine seront davantage discutées dans la section 1.5.4.

La réponse inflammatoire contre *S. suis* est caractérisée par une forte expression de cytokines pro-inflammatoires, telles que le TNF-α, l'interleukine (IL)-1β, l'IL-6 et l'IL-8[37, 38]. Cette réponse, médiée par plusieurs types cellulaires, a pour objectif de limiter la multiplication bactérienne et réduire l'infection. Cependant, la surproduction de cytokines peut également être responsable de dommages aux organes et être néfaste pour l'hôte. Il est proposé qu'une modulation de la réponse inflammatoire efficace serait cruciale pour combattre l'infection à *S. suis*[37]. La surexpression de médiateurs proinflammatoires et l'infiltration de leucocytes seraient également impliqués dans la perméabilisation de la barrière hématoencéphalique[39].

Il a été démontré que différents composants de *S. suis* sont capables d'activer les récepteurs de type Toll ou « Toll-like receptors » (TLR), dont les TLRs 2, 6 et 9[40, 41, 42]. Les composants de la paroi cellulaire tels le peptidoglycane et l'acide téichoique seraient des activateurs majeurs du TLR2 et donc responsables de l'induction de plusieurs cytokines pro-inflammatoires[40]. Les lipoprotéines, également exprimées à la surface, interagissent spécifiquement avec le complexe TLR2/TLR6[41]. Néanmoins, il a été démontré que la grande létalité et les signes cliniques sévères causés par certaines souches plus virulentes (dont le ST7) étaient indépendants de l'activation du TLR2[43]. Ces résultats suggèrent donc que d'autres voies d'activation spécifiques pourraient être impliqués dans le cas d'infections sévères avec des souches de *S. suis* particulièrement virulentes. De plus, un étude réalisée par Auger & al.

(2017) dont je suis co-auteur (**Annexe; Article XI**) a confirmé l'activation des TLRs 7 et 9 par les acides nucléiques de *S. suis,* une fois dans le phagosome.

1.5. Facteurs de virulence exprimés à la surface

Le terme facteur de virulence est un terme relativement large qui porte souvent à confusion. Le terme virulence est une notion quantitative de toute bactérie pathogène. Alors qu'un pathogène se caractérise par la capacité de causer une maladie, la virulence d'un pathogène peut varier à travers les souches d'une même espèce[26]. La présence de facteurs de virulence tels que les adhésines et la CPS contribue à la virulence d'une souche de *S. suis*[28]. Une souche moins virulente possède tout de même le pouvoir pathogène de *S. suis*[44]. La virulence entre différentes souches de *S. suis* est très variable. De plus, la virulence d'une même souche peut également dépendre du modèle expérimental utilisé[44, 45]. Pour ces raisons, la pathogenèse de l'infection causée par *S. suis* est très complexe et beaucoup de mécanismes demeurent à être élucider.

Les facteurs de virulence peuvent être regroupés en trois classes de facteurs. Une analogie intéressante est celle d'un pistolet. Le projectile serait le 'vrai' facteur de virulence', par exemple une toxine ou une adhésine, c'est-à-dire le facteur de virulence de premier plan qui interagit directement sur les cellules ou composants de l'hôte. Il y aurait ensuite le fusil, qui à l'instar des systèmes de sécrétions, permet la sécrétion de la toxine. On peut alors parler de gènes fonctionnels favorisant l'expression des facteurs de virulence. Puis finalement, il y a le tireur, qui ici, serait les gènes nécessaires à la survie et l'adaptation du pathogène, tels les systèmes de régulation, d'acquisition de métaux et de nutriments (métabolisme) ainsi que les systèmes de résistances (toxicité, acidité, peroxyde)[26].

De manière générale, les 'vrais' facteurs de virulence de *S. suis* reposent sur les protéines sécrétées et sur les composants de surface qui interviennent directement dans l'inhibition de la phagocytose (c.à.d., dans l'évasion immunitaire), dans l'adhésion (suivi ou

non d'invasion) aux cellules de l'hôte ou encore, qui modulent la réponse inflammatoire[26, 28]. Ils existent seulement quelques 'vrais' facteurs de virulence critiques pour l'établissement de l'infection chez *S. suis* (**Tableau I**). Les mutants déficients en ces facteurs critiques sont considérés comme avirulents ou ayant une virulence fortement réduite. Plusieurs autres facteurs impliqués dans la virulence, comme les systèmes de régulation et de résistances, ont été caractérisés chez *S. suis* (**Tableau I**). Les mutants de ces facteurs sont toujours virulents, mais leur virulence est partiellement atténuée par rapport à la souche mère. Il existe également des éléments mobiles qui augmentent la virulence de la bactérie. Un exemple concret est la souche responsable de l'épidémie de *S. suis* 2005, en Chine. Cette souche considérée comme hautement virulente possède des facteurs de virulence supplémentaires codés sur un îlot de pathogénicité[46, 47, 48]. Cet îlot code, en autre, pour un système de sécrétion de type IV et des toxines sécrétées par ce même système, dont la toxine « subtilisin-like protease-1 » (**Tableau I**).

Dans ce document, nous nous intéresserons davantage aux facteurs de virulence exprimés à la surface, soit les composants de surface. Les composants de surface tels les protéines de surface et la CPS sont des facteurs de virulence qui interagissent directement avec les cellules de l'hôte, et sont par conséquent, des cibles de choix pour étudier l'impact des composants de surface dans la pathogenèse de l'infection causée par *S. suis*.

1.5.1. Capsule polysaccharidique (rôle dans la virulence)

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 important, selon les études réalisées avec le sérotype 2 (**Figure 2**) [28, 49].

Dans cette section nous détaillerons plus précisément le rôle de la CPS dans la pathogenèse de l'infection causée par *S. suis*. L'organisation génétique, la synthèse et la structure de la CPS seront pour leur part décrites dans la section 2.

Le rôle de la CPS dans la virulence de *S. suis* sérotype 2 a été extensivement étudié dans les dernières années. La CPS exerce plusieurs fonctions lors de l'infection ; (i) un rôle antiphagocytaire[49], (ii) un rôle immunomodulateur[50, 51] et est également capable de (iii) moduler l'adhésion et l'invasion aux cellules de l'hôte[52].

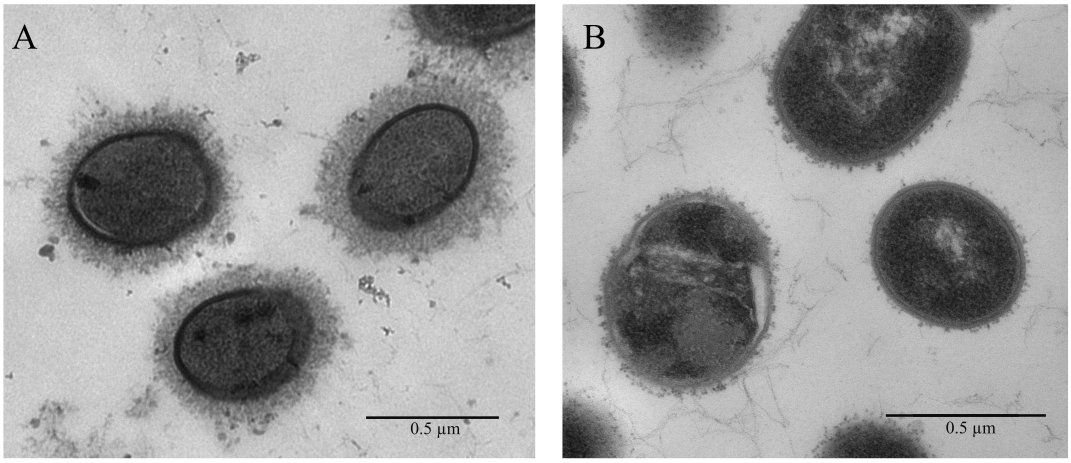


Figure 2. Image de microscopie électronique démontrant la présence de la CPS polysaccharidique à la surface de *S. suis* sérotype 2 (A) et un mutant au phénotype non encapsulé (B). La stabilisation de la CPS à la ferritine permet la visualisation de l'épaisse couche de CPS polysaccharidique qui recouvre *S. suis* (A). Une mutation au sein du locus capsulaire permet d'inhiber l'expression de la CPS, comme démontré en (B).

1.5.1.1. Propriétés antiphagocytaires

La CPS est en premier lieu un facteur antiphagocytaire qui permet à *S. suis* d'éviter les premières lignes de la défense immunitaire de l'hôte, c'est-à-dire les cellules phagocytaires de la réponse immunitaire innée, dont les macrophages et les neutrophiles. Ces cellules sont recrutées au site de l'infection et leur but premier est d'internaliser et détruire les micro-organismes pathogènes.

Pour faire un bref rappel, les cellules du système immunitaire inné reconnaissent les micro-organismes via des récepteurs spécifiques à certains patrons moléculaires bactériens appelés communément « pattern-recognition receptors » (PRRs). Les récepteurs PRRs présents à la surface des cellules du système immunitaire inné reconnaissent des patrons moléculaires bactériens, connus sous le nom de « pathogen-associated molecular patterns » (PAMPs), comme le peptidoglycane, les lipoprotéines, l'acide téichoïque, etc[53].

Dans le cas, par exemple, des macrophages, le contact entre un récepteur PRRs et son ligand PAMPs va induire une cascade de signalisation cellulaire qui activera ultimement le réarrangement du réseau actine. Une particularité de la phagocytose médiée par un contact récepteur-ligand est le regroupement des récepteurs membranaires au site de contact. Les radeaux lipidiques, zone riche en cholestérol, sont des micro-domaines de la membrane plasmique qui permet ce regroupement particulier de récepteurs. Le regroupement progressif des récepteurs permet d'amplifier le signal cellulaire de la phagocytose et de coupler les récepteurs à leurs protéines kinases effectrices. La phosphorylation des phospholipides intermédiaires mène ultimement à l'activation de GTPases qui contrôlent le réarrangement du réseau d'actine nécessaire à la phagocytose[54, 55, 56].

Le mécanisme par lequel la CPS de *S. suis* inhibe la phagocytose a été étudié à l'aide de billes couplées chimiquement à la CPS purifiée de *S. suis* sérotype 2. Il a été démontré que la CPS de *S. suis* sérotype 2 inhibe spécifiquement la traduction des signaux intracellulaires nécessaires à la phagocytose chez les macrophages. De plus, lorsque *S. suis* est en contact avec un macrophage, il a été démontré que la CPS déstabilise les micro-domaines lipidiques, ou radeaux lipidiques, afin d'empêcher la liaison de *S. suis* auprès récepteurs de types PRRs[31].

Il n'a présentement aucune autre étude qui a permis de déterminer si cette capacité à éviter la phagocytose via la déstabilisation des radeaux lipidiques et des signaux intracellulaires était propre à la CPS du sérotype 2, ou cette propriété est partagée au sein des autres sérotypes. Néanmoins, certaines études ont déjà observé *in vitro* l'effet du sérotype sur la capacité antiphagocytaire de la CPS. Il y a été démontré que certains sérotypes, notamment les sérotypes 1 et 2, sont moins internalisés par les cellules dendritiques dérivées de la moelle

osseuse (BmDCs) que les sérotypes 4 et 9. Il est proposé que la composition de la CPS pourrait ainsi influencer les propriétés antiphagocytaires de celle-ci. Cependant, d'autres facteurs tels que l'épaisseur de la CPS et le bagage génétique différent des souches pourraient également influencer les propriétés antiphagocytaires des souches à l'étude[51]. En effet, il a déjà été démontré que différentes souches de *S. suis* expriment une épaisseur variable de CPS[15]. De plus, au moment de d'amorcer cette thèse, l'effet antiphagocytaire de la CPS a pu être validé avec des mutants non-encapsulés uniquement chez le sérotype 2. Le mutant non encapsulé du sérotype 2 est d'ailleurs moins internalisé que les sérotypes 4 et 9 encapsulées suggérant la présence de d'autres facteurs antiphagocytaires chez *S. suis*, d'où l'importance d'avoir des mutants isogéniques partageant le même bagage génétique.

1.5.1.2. Immunomodulation

La CPS possède également un rôle dans la modulation de la réponse inflammatoire. Il a été proposé que la CPS de *S. suis* agit comme bouclier à la surface de la bactérie afin d'envelopper et de cacher les composants de la paroi cellulaire et de diminuer la réponse immunitaire de l'hôte. Des tests *in vitro* avec différents types de cellules, dont les BmDCs et les macrophages, ont permis de démontré qu'une souche mutante non-encapsulée de *S. suis* sérotype 2 induit davantage l'expression de cytokines pro-inflammatoires telles que le TNF-α et l'IL-12p70 que la souche mère encapsulée[57]. L'exposition des composants de la paroi, tels que les lipoprotéines et l'acide téichoïque, via l'activation des TLRs (2 et 6) en serait en majoritairement responsable[51].

Une autre propriété immunomodulatrice de la CPS est sa capacité à induire la production de certaines chimiokines et de médiateurs tels que MCP-1 chez les leucocytes porcins et les BmDCs humains ainsi que la prostaglandine E2 (PGE2) et la métalloprotéinase 9 (MMP-9) chez des cellules différenciées en macrophages[57, 58]. La production de ces deux derniers médiateurs est augmentée en présence des cellules endothéliales microvasculaires du cerveau. Il a été démontré que la chimiokine MCP-1 favorise l'infiltration de leucocytes et déstabilise l'intégrité de la barrière hématoencéphalique, ce qui pourrait permettre à *S. suis*

d'infiltrer le SNC[40]. Quant à eux, l'induction de PGE2 et MMP-9 au niveau de la barrière hématoencéphalique pourrait représenter un facteur critique afin d'augmenter l'étanchéité de la barrière et l'établissement de la méningite[50].

Un fait important est qu'il semble exister des différences dans l'activation des cellules immunitaires entre les différents sérotypes. Les sérotypes 1, 7 et 9 activent davantage les BmDCs que le sérotype 2. Néanmoins, les mêmes remarques précédemment mentionnées subsistent, soit que d'autres facteurs, autre que la composition de la CPS, pourraient influencer les propriétés immunomodulatrices.

De plus, une caractéristique de la CPS de *S. suis* sérotype 2 est qu'elle est très peu immunogénique. Il a été démontré qu'aucun anticorps dirigé contre la CPS de *S. suis* sérotype 2 est présent chez les animaux convalescents suite à l'infection[59, 60] ou chez des souris immunisées avec la CPS purifiée de ce sérotype[61].

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

La CPS peut également moduler l'adhésion et l'invasion des cellules de l'hôte. La CPS affecte directement l'adhésion et l'invasion des cellules en interférant avec l'action des adhésines. Une propriété, qui pourrait sembler désavantageuse, est la diminution du pouvoir d'adhérence de *S. suis* aux cellules de l'hôte en présence de la CPS. L'absence de la CPS favorise l'adhésion de *S. suis* à plusieurs types de cellules épithéliales et endothéliales et semble également favoriser l'invasion des cellules endothéliales microvasculaires du cerveau de porc (pBMEC) ou des cellules épithéliales du larynx humain [52, 62, 63, 64].

L'adhésion étant crucial à la colonisation, l'expression de la CPS pourrait en effet nuire à la colonisation de *S. suis*. Une théorie de plus en plus soutenue est que *S. suis* modulerait l'expression de sa CPS en fonction de l'environnement. L'inhibition de la synthèse de la CPS favoriserait ainsi le contact entre les adhésines de *S. suis* et leurs ligands lors de la

colonisation. Les différents systèmes de régulations impliqués dans l'expression de la CPS seront discutés dans la section 2.4.

1.5.2. Protéines de surface ancrées à la paroi bactérienne

Les protéines de surface peuvent être ancrées à la paroi bactérienne via un lien rattachant la protéine au peptidoglycane. Les protéines destinées à être ainsi ancrées à la paroi contiennent un peptide signal (Leu-Pro-X-Thr-Gly; LPXTG) conservé dans la portion C-terminale de la protéine, suivi par un domaine hydrophobique et une queue positivement chargée[65]. *S. suis* possède plusieurs protéines de surface ancrées à la paroi. Des analyses *in silico* ont déterminés qu'il y avait 33 protéines contenant le peptide signal LPXTG chez *S. suis* 05ZYH33[66]. Nous nous intéresserons plus particulièrement à Sao et aux protéines liant le facteur H car ces protéines ont des rôles encore peu connus dans la pathogenèse de l'infection causée par *S. suis*. Néanmoins, plusieurs autres protéines contenant le signal LPXTG ont été caractérisées comme le « serum opacity-factor » la hyaluronidase, la phosphodiestérase nucléotidique cyclique et la MRP (cette dernière sera discutée dans la section 1.5.4)[67, 68, 69, 70]. Certaines protéines exprimées chez *S. suis,* telles que les pili, sont associées génétiquement à une sortase spécifique [71]. Pour leur part, les protéines de surface Sao, Fhb et Fhbp ne sont pas associées génétiquement à une sortase spécifique et sont ancrées via la sortase A.

1.5.2.1. Sortase A

La sortase est l'enzyme qui catalyse l'attachement des protéines à motif LPXTG au peptidoglycane. Cette enzyme reconnait le motif LPXTG et le clive au niveau de la thréonine (T) et du glycine (G) pour ensuite lier le groupe carboxylé de la thréonine au peptidoglycane[65]. Dans le génome de *S. suis* cinq gènes potentiellement codant des sortases ont été caractérisés, soit *srtABCDE*[72]. Des mutants déficients dans chacun de ces gènes ont permis de déterminer que le gène *srtA,* en contraste avec les autres gènes*,* était nécessaire à l'ancrage de plus d'une quinzaine de protéines de surface[66, 73]. Il a également été démontré que la délétion du gène

srtA résultait en un phénotype avirulent démontrant une capacité diminuée d'adhésion et à coloniser certains organes chez le porc[73].

1.5.2.2. Sao

La protéine « surface antigen-one » ou Sao est une protéine possédant le signal peptide LPXTG et est ancrée à la surface via la sortase A. Cette protéine a été découverte par Li & al. (2006) en recherchant des candidats pour des vaccins sous-unitaires contre *S. suis*[74]. Pour ce faire, l'ADN génomique de *S. suis* a été fragmenté et les fragments clonés dans un vecteur d'expression afin d'être exprimé chez *Escherichia coli*. Les clones d'*E. coli* exprimant différentes protéines clonées ont ensuite été testés par Western Blot avec un sérum de porc en convalescence afin de trouver les protéines susceptibles d'être immunogéniques. Une fois la protéine Sao identifiée, des tests d'immunisation ont démontré son potentiel immunogénique en induisant une forte réponse anticorps IgG chez la souris et le porc. Outre son rôle de protection dans l'infection chez la souris et le porc, il a été démontré que les anticorps dirigés contre Sao augmentent l'opsonophagocytose de *S. suis* par les neutrophiles. De plus, les anticorps spécifiques dirigés contre la protéine Sao réagissent avec des isolats de 28 des 33 sérotypes, ce qui en fait une protéine très intéressante au niveau de développement d'un vaccin sous-unitaire. Au moment d'écrire cette thèse, Sao est toujours une cible d'intérêt dans la recherche d'un vaccin sous-unitaire. En effet, une étude récente a publié des résultats intéressants sur un vaccin composé de bactérines et de la protéine Sao recombinante (rSao). Il a été démontré que l'immunité acquise par la mère par l'administration du vaccin (bactérine+rSao) permet de protéger efficacement les porcelets contre une infection expérimentale avec des souches homologues, mais également hétérologues de *S. suis*[74, 75].

Au sein de la population de *S. suis*, trois variants ou allèles de la protéine Sao ont été identifiés, soit Sao-S; la version la plus courte (gène = 1.5 kb), Sao-M (gène = 1.7 kb) et Sao-L; la version la plus longue (gène = 2.0 kb). Malgré la présence de différents types de variants, la protéine recombinante couplée à un adjuvant confère une protection croisée contre des souches hétérologues de *S. suis* appartenant au sérotype 2. Sao-M est le type le plus

fréquemment retrouvé chez *S. suis* et un test ELISA a d'ailleurs été développé (mais pas encore validé) à des fins d'épidémio-surveillance de *S. suis*[76]. Malgré ces propriétés immunogéniques, le rôle de la protéine Sao dans la pathogenèse de l'infection causée par *S. suis* demeure inconnu.

1.5.2.3. Les protéines Fhbp et Fhb et le facteur H humain

Il existe deux protéines différentes décrites chez *S. suis* capables de lier le facteur H humain, les protéines Fhbp (SSU0186 chez P/17) et Fhb (SSU0253 chez P1/7). La protéine Fhbp est composée de 561 acides aminés alors que la protéine Fhb en compte 765. Ces deux protéines partagent une identité protéique de 33% et sont par conséquent significativement différentes. Les protéines Fhpb et Fhb contiennent une séquence LPXTG nécessaire à la reconnaissance par la sortase A pour l'ancrage à la paroi bactérienne.

Jusqu'à maintenant seulement la protéine Fhb a été caractérisée pour son rôle dans la pathogenèse de l'infection causée par *S. suis*. Il a été démontré qu'un mutant déficient en la protéine Fhb a une résistance diminuée à la phagocytose (en présence de sérum) et une virulence atténuée par rapport à la souche mère dans un modèle *in vivo* porcin[77].

Quant à lui, le gène codant pour Fhbp est exprimé à la hausse chez les bactéries isolées du cerveau et des poumons durant une infection expérimentale de *S. suis* chez le porc[77, 78]. Cependant, aucune étude n'a évalué son rôle dans la virulence de *S. suis*. Néanmoins, cette protéine semble être un facteur immunogénique important chez *S. suis* considérant la production d'anticorps dirigés contre cette protéine chez les porcs infectés[78].

Le gène codant pour la protéine Fhb est retrouvé chez les sérotypes 1, 2, 4, 7 et 9, alors que le gène codant pour la protéine Fhbp est retrouvé chez les sérotypes 1, 1/2, 2, 3 et 5. De plus, des gènes codant pour des protéines avec une identité protéique semblable (%) sont retrouvés chez les sérotypes 7 (45%), 9 (42%) et 14 (100%).

Le facteur H est une protéine régulatrice soluble du complément, produite par l'hôte, et qui protège les cellules de l'hôte des dommages pouvant être causées par la cascade du complément. Le rôle biologique premier de cette molécule consiste à lier le C3b et agir comme cofacteur au facteur-I afin d'initier le clivage protéolytique résultant dans l'inactivation du C3b en iC3b et ainsi protéger les cellules de l'hôte contre son propre complément. Le facteur H est capable de lier plusieurs ligands différents grâce à ces vingt distincts domaines formant cette molécule[79]. Parmi les vingt domaines conservés répétés formant la molécule du facteur H, trois peuvent lier le C3b. De plus, le facteur H agit comme composant de la matrice extracellulaire en liant certains récepteurs cellulaires et certains ligands comme les glycoaminoglycanes et les hydrates de carbone, dont l'acide sialique (**Figure 3**).

Le rôle exact du facteur H est encore nébuleux dans la pathogenèse de *S. suis*. Plusieurs pathogènes sont capables d'exprimer des adhésines pouvant lier le facteur H à leur surface. Parmi ces pathogènes ont retrouve, en autre, *Borrelia burgdolferi* et *pertussis*[80, 81], *Streptococcus pyogenes* et *pneumoniae*[82, 83], *Neisseria meningitidis* et *gonorrhoeae*[84, 85] et *Haemophilus influenzae*[86]. Ces pathogènes tirent profit du recrutement du facteur H à leur surface de deux façons; (i) afin d'augmenter l'adhésion et l'invasion aux cellules de l'hôte[87] et (ii) afin d'échapper au système immunitaire de l'hôte par la résistance au complément[85].

L'adhésine PspC de *S. pneumoniae* est capable de lier plusieurs domaines du facteur H; dont SCR8-10[88, 89], SCR12-15[89] et SCR19-20[88]. Il a été démontré que le recrutement du facteur H à la surface via PspC favorise l'adhésion et l'invasion aux cellules endothéliales et aux cellules épithéliales. Les glycoaminoglycanes de la matrice extracellulaire seraient les composants de l'hôte permettant au facteur H de faire le pont entre la bactérie et la cellule de l'hôte[87]. Les protéines liant le facteur H peuvent donc être considérées comme des adhésines indirectes en contribuant à l'adhésion aux cellules de l'hôte, par contre, leur rôle chez *S. suis* reste à être démontré.

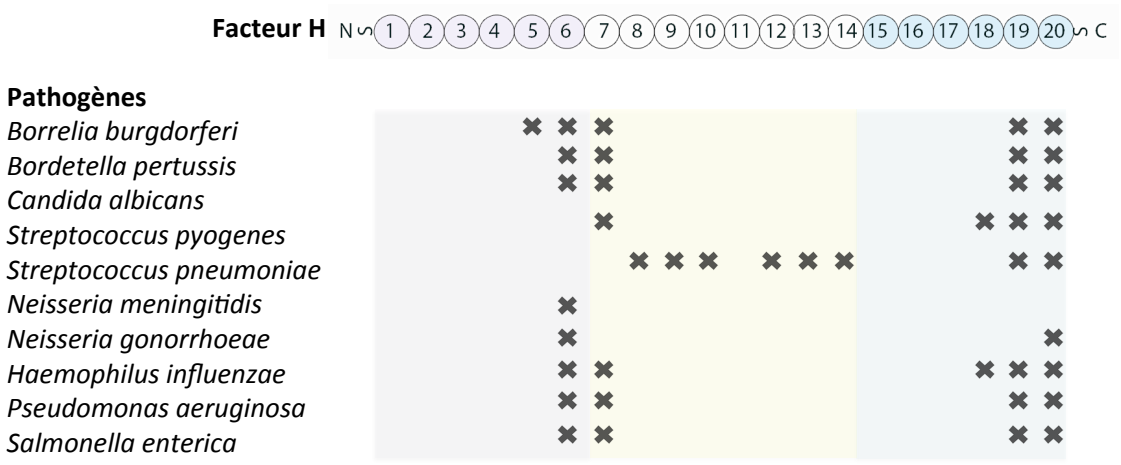


Figure 3. Schéma représentant les différents domaines de liaisons du facteur H utilisés par différents pathogènes. Les régions SCR1-4 sont les régions associées à l'activité du facteur H dans l'inactivation du C3b. Les régions SCR7-20, également utilisées par les pathogènes, sont associées à la reconnaissance de différents ligands (héparine, glycoaminoglycanes, acide sialique)[90]. Les sites illustrés pour les pathogènes sont les sites utilisés par une ou plusieurs protéines liant le facteur H situé(s) à la surface de la bactérie. Figure modifiée à partir de Jozsi & al. (2015)[90].

Un autre avantage du recrutement du facteur H à la surface de la bactérie, dont certains pathogènes utilisent pour échapper au système immunitaire de l'hôte, est la résistance au complément conféré par le facteur H. Un exemple est le pathogène *N. meningiditis* qui recrute le facteur H via la liaison du domaine SCR6 par la porine Por1A. Une fois à la surface, le facteur H peut ainsi lier le C3b et le cliver en sa forme inactive l'iC3b, qui demeure attaché à la surface de la bactérie[85]. De plus, dans le cas de *S. pneumoniae*, la résistance au complément est proportionnelle à la quantité de facteur H recrutée à la surface de la bactérie via la protéine PspC[91].

1.5.3. Autres adhésines

La colonisation des voies respiratoires supérieures du porc par *S. suis* et sa dissémination subséquente nécessitent une interaction avec les composants de la matrice extracellulaire et des cellules épithéliales et endothéliales[28]. La matrice extracellulaire est une

structure interreliée complexe composée de différentes fibres/protéines dont le collagène, la fibronectine, la laminine, l'élastine et de divers glycoaminoglycanes tels l'héparine et l'héparane sulfate[92].

Le génome de *S. suis* code pour plusieurs adhésines, protéines et antigènes ayant des propriétés adhésives dont certaines ayant des fonctions similaires ou des ligands identiques (voir **Tableau I**). Plusieurs dizaines de protéines ont été décrites comme ayant des propriétés d'adhésion chez *S. suis*, cependant, dans ce document, nous ciblerons seulement quelques exemples d'adhésines importantes impliquées la virulence de *S. suis*. Un tableau plus complet listant l'ensemble des adhésines et autres facteurs de virulence proposés à ce jour est disponible à des fins de références (**Tableau I**).

1.5.3.1. Cbp40; Protéine liant le collagène

Le collagène est la protéine la plus abondante du corps humain. Elle est sécrétée par les cellules des tissus conjonctifs (majoritairement les fibroblastes) dans la matrice extracellulaire sous forme de tropocollagène où celui-ci est ensuite polymérisé en fibre de collagène[92]. Plusieurs pathogènes, dont *Streptococcus mutans*[93] et *S. pneumoniae*[94], ont la capacité de lier le collagène via des adhésines afin de favoriser la colonisation.

Chez *S. suis,* une protéine possédant une homologie avec une adhésine de *S. aureus* liant le collagène a été clonée et caractérisée. La protéine, nommée Cbp40, a été initialement caractérisée comme facteur de virulence potentiel en utilisant la technique « suppression subtractive hybridization », qui permet de cibler des gènes associés à des souches virulentes. Les propriétés adhésives de la protéine pour le collagène ont ensuite été démontrées par ELISA et western-blot. Une propriété intéressante de cette protéine est qu'elle semble immunogénique chez le porc et confère une protection contre l'infection à *S. suis* lors d'infections expérimentales chez le poisson-zèbre « zebrafish ». Néanmoins, aucune donnée n'est disponible sur la distribution de cette protéine dans les souches de *S. suis* et si elle permet une protection croisée contre d'autres sérotypes/souches.

Une réduction dans l'adhérence aux cellules épithéliales HEp-2 et une diminution de la virulence lors d'infection expérimentale chez le poisson-zèbre ont été observées chez une souche mutante déficiente en la protéine Cbp40, suggérant un rôle significatif de la liaison au collagène via cette protéine dans la colonisation de *S. suis*. De plus, il a été démontré que la protéine Cbp40 contribue à la production de cytokines impliquées dans le recrutement de neutrophiles par les cellules endothéliales. Il est suggéré que l'adhésion au collagène et l'induction de cytokines au niveau de la barrière hématoencéphalique par Cbp40 contribuerait à la progression de l'infection en méningite[95].

1.5.3.2. Fbps; Protéine liant la fibronectine

La fibronectine est une glycoprotéine structurale qui intervient dans le contrôle de la disposition du collagène dans la matrice extracellulaire et dans l'attachement de la cellule au matériel extracellulaire[92]. La protéine recombinante Fbps de *S. suis* est capable de lier la fibronectine, mais également le fibrinogène. Tout comme Cbp40, la protéine Fbps est immunogénique chez les porcelets, mais celle-ci est présente dans tous les 'vrais' sérotypes de *S. suis* et représente donc un candidat intéressant pour un vaccin sous-unitaire offrant une protection croisée pour tous les sérotypes. De plus, Fbps contribue à l'adhérence de la bactérie aux cellules épithéliales et à la production de cytokines pro-inflammatoires par ces cellules. Un mutant déficient en Fbps démontre une diminution dans la dissémination dans certains organes cibles par rapport à la souche mère, suggérant une baisse de virulence chez le mutant Δ*fbps*[96, 97].

1.5.3.3. Énolase; Protéine liant la fibronectine et le plasminogène

La protéine énolase présente chez *S. suis* a été isolé par chromatographie d'affinité à la fibronectine puis séquencé afin de localiser la séquence nucléotidique codante dans le génome de *S. suis*. L'énolase est une protéine impliquée dans la glycolyse et la néoglucogenèse catalysant la conversion du 2-phosphoglycérate en phosphoénolpyruvate. L'énolase ne

possède pas de signal peptide LPXTG pour l'ancrage à la membrane. Chez *S. suis*, on retrouve cette protéine dans le cytosol, mais également dans le milieu extracellulaire et à la surface de *S. suis*[98, 99].

Outre sa capacité à lier le plasminogène - une enzyme protéolytique initiant la fibrinolyse dans le sang - la protéine recombinante énolase est également capable de lier le fibrinogène comme démontré par ELISA. De plus, des expériences d'inhibition menées avec des anticorps dirigés contre la protéine énolase ont permis de mettre en lumière l'implication de cette protéine dans l'adhésion aux cellules pBMEC. Une seconde étude a démontré que la protéine recombinante énolase était capable de lier également aux cellules épithéliales HEp-2[99].

À l'instar de la protéine Fbps, l'énolase est présente chez toutes les souches de référence des différentes sérotypes de *S. suis*. Il a été démontré que la protéine recombinante purifiée énolase possédait des propriétés antigéniques protectrices lors d'une infection expérimentale à *S. suis* chez la souris et pourrait représenter un candidat intéressant pour un vaccin sous-unitaire[99].

1.5.3.4. Pili

Les pili jouent un rôle crucial dans la colonisation de plusieurs pathogènes, favorisant l'adhésion à des tissus spécifiques de l'hôte. Plusieurs types de pili ont été caractérisés chez les bactéries à Gram positif et sont encodés par des locus spécifiques. Les gènes codant pour la synthèse et l'assemblage d'un type de pilus sont encodés au sein du même locus. Parmi ces gènes, on y retrouve (i) le gène codant pour la sous-unité majeure du pilus formant la base du pilus, (ii) le gène codant pour la sous-unité mineure du pilus formant la queue du pilus, (iii) le gène codant pour la sous-unité formant l'extrémité du pilus et (iv) une sortase spécifique au type de pilus. La base du pilus est formée par la polymérisation de sous-unités majeures du pilus à laquelle une ou plusieurs sous-unités mineures sont rattaché(es) afin de former la structure pilaire. Les sous-unités formant la base du pilus demeurent ensuite ancrées dans le

peptidoglycane via leur séquence signal LPxTG en C-terminal (grâce à l'action d'une sortase). Le pilus peut être formé seulement de la base du pilus et en absence des sous-unités; néanmoins, des mutations dans le gène codant pour la sous-unité majeure du pilus inhibe la formation du pilus[100, 101, 102].

Chez *S. suis*, quatre loci potentiels ont été caractérisés par bio-informatique; ***srtBCD*, *srtE*, *srtF*, *srtG*.** Au sein de la population de *S. suis*, on répertorie 12 génotypes différents (A à L) basés sur la présence ou non de certains loci. Cependant, les génotypes A et B représentent les génotypes les plus isolés dans le cas de mortalité causée par une infection à *S. suis* chez l'humain et le porc. À titre de comparaison, les souches virulentes P1/7 et 31533 (fréquemment utilisées dans la littérature) appartiennent au génotype A, qui est caractérisé par la présence des loci *srtBCD* et *srtF,* alors que la souche 89/1591 (une souche Nord-Américaine à virulence intermédiaire) appartient au génotype B qui est caractérisé par la présence des loci *srtBCD*, *srtF* et *srtG*[71].

Le pilus codé par le locus *srtBCD* ne semble pas être assemblé et exprimé à la surface de *S. suis* dû à la présence de mutation. Cependant, il a été démontré grâce à différents mutants que la sous-unité majeure tronquée (Sbp2) du pilus peut néanmoins agir comme adhésine et être impliquée dans la pathogenèse de l'infection. En effet, il a été démontré que la protéine recombinante Sbp2 lie certains composants de la matrice extracelluaire, dont la fibronectine et la laminine. De plus, des essais d'inhibitions d'adhésion ont permis d'observer une diminution de l'adhésion de *S. suis* lorsque les cellules sont préincubées avec la protéine rSbp2, démontrant le rôle de Sbp2 dans l'adhésion de *S. suis*. L'absence de la sous-unité majeure Sbp2 chez un mutant isogénique diminue l'adhésion de *S. suis* aux cellules épithéliales HEp-2 et diminue également la virulence de *S. suis* lors d'infection expérimentale chez le poisson-zèbre[103, 104].

Le pilus codé par le locus *srtF* est quant à lui bien exprimé à la surface de *S. suis* et connu sous le nom de 'pilus F'. Une caractéristique de ce pilus est qu'il est formé par la sous-unité principale seulement. Un mutant déficient dans l'expression de la sous-unité Sfp1 est incapable d'exprimer le pilus F à la surface de *S. suis*. Curieusement, l'absence du pilus F chez

la souche mutante n'a pas affecté les propriétés d'adhésion de la souche aux cellules pBMEC. De plus, il a été observé que le mutant du pilus F est aussi virulent que la souche mère dans une infection expérimentale chez la souris[105].

Quant au pilus codé par le locus *srtG*, il a été démontré que ce pilus était bien exprimé à la surface de *S. suis* et que la sous-unité mineure Sgp2 était nécessaire à son assemblage. Il s'agit d'une caractéristique de ce type de pilus, car généralement le pilus peut se former en l'absence des sous-unités mineures. Cependant, le rôle de ce pilus dans la pathogenèse de *S. suis* n'a toujours pas été étudié[106]. Aucune étude n'a également caractérisé l'assemblage ou le rôle du pilus potentiellement codé par le locus *srtE*.

1.5.4. Autres facteurs importants; les marqueurs de virulence

La présence de certaines protéines exprimées par *S. suis* est corrélée avec la virulence de certaines souches. Bien que cette corrélation ne soit pas absolue pour toutes les souches, la plupart des souches virulentes présentent un patron similaire dans l'expression de ces facteurs. En effet, la majorité des souches virulentes de *S. suis* isolés de cas cliniques humains et porcins expriment la plupart du temps les gènes codant pour l'expression de la suilysine (SLY), de la protéine extracellulaire ou facteur extracellulaire (EF) et de la MRP^3. De plus, comme mentionné précédemment, les facteurs MRP et EF sont également présents chez la plupart des souches du complexe allélique ST1, un type allélique fortement associé aux souches causant la méningite et la septicémie. À l'opposé, ces marqueurs sont souvent absents des souches appartenant aux ST25/28[22]. Ces marqueurs sont considérés comme des marqueurs de virulence étant donné que les mutants isogéniques déficients en ces facteurs sont aussi virulents que leur souche mère respective[107, 108, 109, 110].

1.5.4.1. La suilysine

La SLY est une hémotoxine d'environ 54 kDa qui est sécrétée dans le milieu par *S. suis*. La SLY de *S. suis* fait partie d'une famille de toxines appelée « thiol-activated toxins ».

Ces types de toxines sont produits par les bactéries à Gram positif et représentent une famille de cytolysines dont la modification biochimique d'une cystéine inactive l'action de la toxine. La suilysine cible spécifiquement le cholestérol des membranes lipidiques comme récepteur et forme un pore dans la membrane. Le cholestérol libre est d'ailleurs un inhibiteur de l'action de cette toxine[111, 112].

La présence du gène codant la SLY, ainsi que ceux des gènes *mrp* et *ef*, est corrélée avec la grande virulence des souches de *S. suis* sérotype 2[109]. Comme mentionné précédemment, le type allélique ST1 est également fortement associé au génotype $MRP^{+}EF^{+}SLY^{+}$. Néanmoins, la littérature est contradictoire à savoir si la SLY est un facteur de virulence critique pour *S. suis*[113].

Il a été démontré que la toxine SLY est toxique pour plusieurs types cellulaires, notamment les cellules épithéliales et endothéliales, mais également pour certaines cellules du système immunitaire dont les monocytes et les neutrophiles[114]. La SLY serait également impliquée dans l'invasion et l'adhérence aux cellules épithéliales, la perte des cellules ciliées et l'apoptose des cellules. De plus, la SLY jouerait un rôle dans la résistance au complément comme démontré par Lecours & al. (2011) avec des cellules BmDCs murines[115]. Cette dernière étude a également démontré le rôle de la SLY dans le relâchement de cytokines immunomodulatrices telles que IL-12p70 et IL-10 par les BmDCs[115]. D'autres études *in vitro* ont démontré que la protéine recombinante SLY induit le relâchement de cytokines pro-inflammatoires (TNF-α et IL-6) par les macrophages et monocytes, démontrant le rôle important de cette toxine dans la modulation de la réponse immunitaire[116].

Néanmoins, il a été démontré que la seule présence du gène *sly* n'est pas suffisante pour qu'une souche de *S. suis* soit cytotoxique pour les cellules de l'hôte. En effet, une étude a rapporté que le niveau de transcription du gène *sly* par son promoteur jouait un rôle majeur dans la virulence et la pathogenèse d'une souche. Les observations ont permis de mettre en évidence que les souches ST1 expriment davantage le gène *sly* ce qui mène à une plus grande production de SLY et une plus grande cytotoxicité par rapport à des souches sly^{+} appartenant au type allélique ST104. Les auteurs de cette étude concluent également qu'une plus grande

expression du gène *sly* chez les souches ST1 favoriserait le développement de la méningite[36].

Malgré les rôles proposés pour la SLY chez les souches virulentes de *S. suis*, la contribution de la suilysine à la pathogenèse de l'infection causée par *S. suis* est contradictoire dans la littérature. En effet, lors d'une infection expérimentale chez le porc par aérosol, qui reproduit la voie d'infection naturelle de *S. suis,* il a été observé que la production de la SLY n'était pas cruciale à la virulence de *S. suis* étant donné que les souches déficientes dans la production de cette toxine conservent leur pouvoir pathogène[107]. La SLY ne semble pas être un facteur de virulence critique dans ce modèle utilisé.

Une caractéristique intéressante de la SLY est que le gène codant cette protéine (*sly*) est hautement conservé au sein des souches de *S. suis*. Il a été démontré que le gène *sly* était homologue à 99,5% entre la souche Nord-Américaine SX332 et la souche Européenne 31533. Cette haute homologie conservée entre des souches géographiquement distinctes en fait un candidat intéressant pour un vaccin sous-unitaire, au moins contre les souches SLY+. D'ailleurs, il a été démontré que la SLY est très immunogénique. L'immunisation avec la protéine recombinante SLY protège contre une infection avec une dose létale dans un modèle d'infection murin. De plus, cette même protéine permet d'induire une protection partielle contre *S. suis* lors d'une infection expérimentale chez le porc[36, 107].

1.5.4.2. Le facteur extracellulaire (EF) et la « Muraminidase-released protein » (MRP)

Deux autres protéines servent souvent comme marqueurs de virulence chez *S. suis*; la protéine MRP et le facteur EF. La protéine MRP est une protéine membranaire de 136 kDa qui est également relâché dans le milieu. MRP est immunogénique chez le porc et est conservée chez la plupart des souches asiatiques et européennes, ce qui en fait un candidat vaccinal intéressant pour ces régions. Six variants différents de *mrp* (136 kDa) ont été décrit comme étant présents au sein de la population de *S. suis*. Le premier variant, appelé mrp^{s}, code pour la forme courte de la protéine (environ 120 kDa), alors que les variants *mrp** à mrp^{****} correspond aux variants de plus grande masse (>136 kDa). La protéine Mrp^{s} produite est

tronquée à l'extrémité *N*-terminale de la protéine par rapport à la forme native de Mrp. Les variants *mrp*^{s} et *mrp** sont les variant les plus isolés. Il a également été démontré par immunobuvardage que les variant codés par les gènes *mrp**** et *mrp***** ne sont pas exprimés chez les souches testées de *S. suis*. Le variant *mrp**** est particulièrement retrouvé chez les ST25, alors que les ST1 possèdent pour la plupart le gène *mrp* normal[109, 117].

Curieusement, il a été rapporté que la protéine MRP possède la capacité de lier le fibrinogène humain, ce qui augmenterait les propriétés antiphagocytaires de *S. suis*. Il a été démontré que la portion N-terminale de MRP est capable de lier le fibrinogène afin de créer un pont avec les récepteurs de type intégrine sur les leucocytes et d'ainsi moduler leurs fonctions, permettant à *S. suis* de mieux survivre dans le sang. De plus, MRP contribuerait à augmenter la perméabilité de la barrière hématoencéphalique. Néanmoins, les mécanismes exacts de ces phénomènes sont encore inconnus [108, 118]. D'ailleurs, une étude récente semblerait associer la MRP à une panoplie des fonctions, difficilement performées par une seule et même protéine. Ainsi, le vrai rôle de la MRP dans la pathogenèse de l'infection demeure contradictoire[119].

Quant à lui, le facteur EF, est une protéine de 110 kDa sécrétée par *S. suis*. Tout comme MRP, EF est également immunogénique et conservé entre les souches de *S. suis*. Il existe un variant du facteur EF, EF* qui présente une masse supérieure à 110 kDa. Il existe donc différents profils pour MRP et EF. Il a été observé que la majorité des souches MRP^{+}/EF^{+} sont isolées à partir de porcs malades, notamment en Europe et Asie, alors que les souches MRP^{-}/EF^{-} sont souvent isolées à partir des amygdales de porcs sains (porteurs sains), suggérant une différence dans la virulence de ces souches. En contraste, les souches de sérotype 2 isolées en Amérique du Nord, considérées comme moins virulentes, démontrent majoritairement un profil MRP^{-}/EF^{-}[22, 108].

Néanmoins, des mutants déficients en ces protéines sont aussi virulents que leur souche mère et possèdent toujours la capacité à causer une infection, ce qui suggère que MRP et EF ne sont pas des facteurs de virulence critiques pour la pathogenèse de l'infection causée par *S. suis*. De plus, les souches de sérotype 2 Nord-Américaines, isolées de cas de méningite ou septicémie chez l'humain ou le porc sont MRP^{-}/EF^{-}. Cette observation démontre que MRP

et EF ne sont pas nécessaires à l'établissement de l'infection par *S. suis*. Une autre observation qui suggère que le facteur EF n'a pas de rôle dans la virulence, et ce, indépendamment du sérotype, est que les souches de sérotypes 7 et 9 isolées de tissus de porcs infectés en Europe sont EF négatives[117]. Comme la SLY, la présence des gènes *mrp* et *ef* ne semble pas suffisante pour confirmer l'expression de la protéine. Une étude avec des souches isolées aux États-Unis a en effet démontré que des mutations dans le gène *mrp* pouvaient inhiber la production de la protéine[120]. De ce fait, les associations entre génotype et virulence doivent être interprétées avec précaution.

Tableau I. Facteurs de virulence proposés ou confirmés impliqués dans la pathogenèse de l'infection causée par *S. suis*

Facteur[a]	Fonction[b]	Virulence du mutant	Références
Régulation			
1910KH/RR	Système de régulation à deux composants	Atténuée (porc)	121
AdcR	Régulation de l'acquisition du zinc	Atténuée (souris)	122
ArcD	Arginine-ornithine antiporteur (système arginine deiminase)	Mutant non testé	123
ArgR	Régulateur transcriptionnel de arcBCD (système arginine deiminase)	Mutant non testé	124
Ccpa	Régulateur transcriptionnel du *'carbon catabolite repressor*'	Atténuée (souris)	125, 126
CiaRH	Système de régulation à deux composants	Atténuée (souris)	127
CodY	Protéine régulatrice globale	Atténuée (souris)	128
CovR	Protéine régulatrice	Augmentée (porc)	129
Fur	Régulation de l'acquisition du fer	Atténuée (souris)	122
Homologue à *S. mutans* SMU_61	Régulateur transcriptionnel	Atténuée (porc)	130
IhK/Irr	Système de régulation à deux composants	Atténuée (souris)	131
NadR	Régulateur transcriptionnel	Atténuée (porc)	130
NisK/NisR	Système de régulation à deux composants	Atténuée (souris)	132
RevS	Protéine régulatrice	Atténuée (souris)	133
RevSC21	Protéine régulatrice	Atténuée (porc)	134
Rgg	Régulateur transcriptionnel	Atténuée (porc)	135
Rss04	Petit ARN (régulateur transcriptionnel)	Atténuée (souris)	136
SalK/SalR	Système de régulation à deux composants	Avirulent (porc)	137
Stk	Sérine thréonine kinase	Atténuée (porc/souris)	138
Tran	Régulateur transcriptionnel	Atténuée (poisson-zébre)	139
TreR	Régulateur transcriptionnel	Atténuée (porc)	130

Facteurs exposés à la surface et protéines sécrétées			
6-phosphogluconate-dehydrogenase	Adhésion cellules épithéliales	Mutant non disponible	[140]
Abpb	Arginine peptidase	Atténuée (souris)	[141]
ApuA	Pullulanase	Mutant non testé	[142]
Atl	Autolysine	Atténuée (poisson-zébre)	[143]
BgaC	Béta-galactosidase	Identique (souris)	[144]
Cbp40	Adhésion au collagène type-I	Atténuée (poisson-zébre)	[95]
Collagenase	Dégradation du collagène	Atténuée (porc)	[130]
Collagenase-like protéase	Adhérence et invasion de la barrière hématoencéphalique	Mutant non disponible	[145]
CPS[c]	Capsule polysaccharidique	Avirulent (porc/souris)	[49, 146]
Dipeptidylpeptidase IV (DppIV)	Adhésion à la fibronectine	Atténuée (souris)	[147]
DltA	D-alanylation de l'acide téichoique	Atténuée (porc)	[115, 148]
EF	Facteur extracellulaire	Identique (porc)	[108]
Endo-β-N-acétylglucosaminidase D	Dégradation des oligosaccharides de l'hôte	Atténuée (porc)	[130]
Enolase	Adhésion à la fibronectine, au plasminogène et au collagène	Mutant non disponible	[98, 149, 150, 151]
Fbps	Adhésion à la fibronectine	Atténuée (porc)	[96, 152]
Fhb	Protéine liant le facteur H humain	Atténuée (porc)	[77]
Fhbp	Protéine liant le facteur H humain	Identique (souris)	Données non publiées
GAPDH	Adhésion cellules épithéliales	Mutant non disponible	[153, 154]
gène SSU0587	Béta-galactosidase	Identique (souris)	[155]
Hhly3	Hémolysine-III	Atténuée (poisson-zébre)	[156]
Homologue SAG0907	Lipoprotéine	Identique (porc)	[130]
Htps	Histidine triad protéine	Mutant non disponible	[157]
IdeSsuis	IgM protéase	Identique (porc)	[32, 158]
IgA1	IgA protéase	Atténuée (porc)	[159]
Igde	IgG protéase	Mutant non disponible	[34, 160]
Lpp	Lipoprotéine	Atténuée (porc)	[130]
Mac	IgM (porcin) protéase	Identique (porc, souris, poisson-zébre)	[161]
MRP	Adhésion au fibrinogène	Identique (porc)	[108]
Pgda	N-déacétylation du peptidoglycan	Atténuée (porc)	[162]
Pilus (srtBCD Pilus)	Adhésion cellules épithéliales (sous-unité Sbp2)	Atténuée (poisson-zébre)	[103]
Pilus (srtE Pilus)	Adhésine potentielle	Mutant non disponible	[71]
Pilus (srtF Pilus)	Adhésine potentielle	Identique (souris)	[105]
Pilus (srtG Pilus)	Adhésine potentielle	Mutant non disponible	[71, 106]
Protéine membranaire potentielle (CP003922.1)	Adhérence et invasion de la barrière hématoencéphalique	Mutant non disponible	[145]
Serum Opacity Factor	Opacification du sérum	Atténuée (porc)	[68]
SrtA	Sortase	Identique (souris)/ Atténuée (porc)	[66, 73]
Ssa	Adhésion à la fibronectine	Atténuée (souris)	[163]
SsnA	DNase	Atténuée (souris)	[164]
SspA (SSU0757)	Protéase	Atténuée (porc/souris)	[165, 166, 167]
SspA-1 (du SST4)	Protéase	Atténuée (souris)	[168]

SsPep	Protéine extracellulaire	Atténuée (porc)	[169]
Streptococcal adhesin P SadP	Adhésion au galactosyl-α1–4-galactose	Mutant non disponible	[170]
Suilysin	Hémolysin	Identique (porc)	[107, 110]
Surface-anchored protein	Adhérence et invasion de la barrière hématoencéphalique	Mutant non disponible	[145]
VirB1-89K	Composant du système du SST4	Atténueé (souris)	[171]
VirB4-89K	Composant du système du système de sécrétion de type 4 (SST4)	Atténueé (souris)	[48]
VirD4-89K	Composant du système du SST4	Atténueé (souris)	[46, 172]
Résistance			
Dpr	Résistance à la toxicité (Fer)	Mutant non disponible	[173]
Nox	Tolérance stress oxidatif	Atténuée (porc/souris)	[174]
PerR	Protéine de la famille 'Fur'	Atténuée (souris)	[175]
SodA	Résistance à la toxicité (superoxide dismutase)	Mutant non disponible	[176]
Spx1	Tolérance au stress	Atténuée (souris)	[174, 177]
Spx2	Tolérance au stress	Atténuée (souris)	[174, 177]
Système Arginine Déiminase	Résistance à l'acidité	Mutant non testé	[178, 179]
Tig	'Trigger factor' / Tolérance au stress (thermique, oxidatif et acidité)	Avirulent (souris)	[180]
Zur	Résistance à la toxicité (Zinc)	Mutant non testé	[181]
Facteurs impliqués dans le métabolisme et transport			
3-kétoacyl-ACP réductase	Adhésion à la fibronectine	Mutant non disponible	[151]
Abpb	Amylase-binding protein B	Atténue (souris)	[141]
Adénylosuccinate synthétase	Adhésion à la fibronectine	Mutant non disponible	[151]
Cdd	Cytidine déaminase	Atténuée (porc)	[130]
Chaperonine GroEL	Adhésion à la fibronectine	Mutant non disponible	[151]
DHH family protein	Adhérence et invasion de la barrière hématoencéphalique	Mutant non disponible	[145]
DivIVA	Adhésion aux cellules épithéliales	Mutant non disponible	[182]
DNA-directed polymerase subunit RNA beta	Adhérence et invasion de la barrière hématoencéphalique	Mutant non disponible	[145]
DnaJ	Adhésion aux cellules épithéliales	Mutant non disponible	[183]
DnaK	Adhésion aux cellules épithéliales	Mutant non disponible	[182]
EF-Tu	Adhésion aux cellules épithéliales	Mutant non disponible	[184]
FeoB	Transporteur (Fer)	Atténué (souris)	[185]
Fhs	Formate-tetrahydrofolate ligase	Atténuée (porc/souris)	[186]
Fructose biphosphate aldolase	Adhésion à la fibronectine et au collagène	Mutant non disponible	[151]
GdpP	c-di-AMP phosphodiestérase	Atténué (souris)	[69]
GidA	Glucose-inhibited division protein	Atténuée (souris)	[187]
GlnA	Glutamine synthétase	Atténué (souris)	[188]
Glutamate dehydrogénase	Adhésion à la fibronectine	Mutant non disponible	[151, 189]
GtfA	Sucrose phosphorylase	Atténuée (porc)	[130]
GuaA	GMP synthétase	Identique (porc)	[130]
HtpsC	Adhésion aux cellules épithéliales	Atténue (souris)	[190]

IMPDH	Inosine 5-monophosphate déshydrogénase	Atténue (souris)/ Avirulent (porc)	[191]
IS630-Spn1	Adhérence et invasion de la barrière hématoencéphalique	Mutant non disponible	[145]
Lactate déshydrogénase	Adhésion aux cellules épithéliales	Mutant non disponible	[184]
Lgt	Maturation des lipoprotéines	Identique (souris)	Données non publiées
Lipoprotein 103	Acquisition du zinc	Atténué (souris)	[192]
Lipoprotein signal peptidase	Exportation des lipoprotéines	Identique (porc)	[193]
LuxS	Quorum sensing	Atténué (poisson-zébre)	[194, 195, 196]
ManN	Transport spécifique du mannose	Atténuée (porc)	[130]
MsmK	ATPase du transporteur de type ABC	Atténuée (souris)	[197]
O-acétylhomosérine sulfhydrylase	Adhérence et invasion de la barrière hématoencéphalique	Mutant non disponible	[145]
Oligopeptide-binding protein OppA precursor	Adhésion à la fibronectine et au collagène	Mutant non disponible	[151]
Perméase	Transporteur de type ABC (acide aminé)	Atténuée (porc)	[130]
Perméase (SSU0835)	Transporteur de type ABC (antibiotique)	Atténuée (porc)	[198]
Phosphoglycérate mutase	Adhésion à la fibronectine et au collagène	Mutant non disponible	[151]
Phospholipase C	Modulation de la production d'acide arachidonique	Mutant non disponible	[199]
Phosphopantothenoyl cystéine décarboxylase	Adhérence et invasion de la barrière hématoencéphalique	Mutant non disponible	[145]
Potentielle glycogène-phosphorylase	Adhérence et invasion de la barrière hématoencéphalique	Mutant non disponible	[145]
PurA	Adénylosuccinate synthétase	Atténuée (porc)	[130]
PurD	Phosphoribosylamine-glycine ligase	Atténuée (porc)	[130]
Pyruvate déshydrogénase composant E1, sous-unité alpha	Adhésion à la fibronectine	Mutant non disponible	[151]
Pyruvate kinase	Adhésion à la fibronectine et au collagène	Mutant non disponible	[151]
ScrB	Sucrose-6-phosphate hydrolase	Atténuée (porc)	[130]
ScrR	Répresseur de l'opéron du sucrose	Atténuée (porc)	[130]
Signal transduction histidine kinase	Adhérence et invasion de la barrière hématoencéphalique	Mutant non disponible	[145]
SsTGase	Glutamine-glutamyltransférase	Atténuée (porc)	[200]
Stp	Sérine/théonine phosphatase	Atténuée (souris)	[201]
Trag	Protéine impliquée dans le transfert d'ADN (SST4)	Atténué (poisson-zébre)	[202]
Translation elongation factor G	Adhésion à la fibronectine et au collagène	Mutant non disponible	[151]
TroA	Acquisition du manganèse	Avirulent (souris)	[203]
Autres			
Gene homologue à spr1018 (*S. pneumoniae*)	Inconnu	Atténuée (porc)	[130]
Hp0197	Inconnu/antigène de surface	Atténuée (souris)	[204, 205, 206]

Sao	Inconnu/antigène de surface	Identique (souris)	[207]
VirA	Inconnu	Atténuée (lapin)	[208]

[a] Le nom du facteur est nommé dans le tableau selon le nom du gène muté ou de la protéine déficiente (par exemple; le gène muté *purA* est cité comme facteur PurA)
[b] Fonctions proposées ou confirmées dans la pathogenèse de l'infection
[c] Le facteur CPS représente l'ensemble des mutants des gènes du locus de la CPS (phénotype identique)

Pour conclure, le concept de 'facteur de virulence' est également vague dans la littérature et mériterait d'être mieux définie chez le pathogène *S. suis*. Depuis 2005, le nombre d'articles scientifiques caractérisant des facteurs impliqués dans la virulence de *S. suis* ont presque quadruplés, passant de 44 (1995-2005) à 172 (2005-2015), démontrant l'urgence d'avoir une notion claire de facteurs de virulence. À titre d'exemple, certains de ces facteurs, notamment ceux impliqués dans le métabolisme, sont peut-être identifiés comme des facteurs de virulence à tort, puisqu'ils ne contribuent pas à la virulence directement, mais à la survie de la souche (ex. acquisition de nutriments). De plus, certaines protéines, comme les adhésines, peuvent avoir un impact sur l'adhésion à certains types de cellules, sans être importantes ou critiques pour la virulence, car leurs fonctions sont compensées par des autres protéines ayant un rôle similaire (redondance de fonctions). Finalement, comme le démontre un article dont j'ai contribué en tant que coauteur (**Annexe; Article XII**), le rôle attribué à certains facteurs peut varier selon le patron génétique de la souche utilisée ou encore du modèle expérimental utilisé.

Certains sérotypes, certains types alléliques et certains facteurs de virulence peuvent être associés à la virulence. Néanmoins, cela ne semble pas être une règle absolue, et la présence d'un seul facteur ne semble pas être suffisante afin de décrire une souche comme étant virulente. De plus, la possibilité de co-infections peut également influencer le potentiel d'une souche à infecter son hôte.

Dans le cas des épidémies à *S. suis* en Chine, l'émergence d'une souche hautement virulente semble indiquer que certains facteurs, lorsqu'exprimés ensemble, peuvent significativement contribuer à la grande virulence d'une souche. En effet, la présence de facteurs de virulence uniques chez *S. suis* codés au sein d'un îlot de pathogénicité sont responsables de la haute virulence de cette souche.

Il semble de plus en plus évident que la virulence d'une souche de *S. suis* dépend de plusieurs facteurs et que la virulence d'une souche ne peut pas être évaluée en considérant qu'un seul facteur. La disponibilité de séquençage des génomes permettra sans doute de mieux évaluer

l'ensemble de ces facteurs, à grande échelle, afin de mieux prédire le potentiel de virulence d'une souche.

1.6. Approches prophylactiques

Aucun vaccin efficace à 100% n'est présentement disponible pour protéger contre une infection à *S. suis*[59]. Plusieurs défis persistent afin d'obtenir un vaccin intéressant. Le vaccin idéal devra induire des anticorps opsonisants et conférer une protection croisée efficace contre plusieurs sérotypes. La vaccination des truies afin de conférer une immunité passive aux porcelets donnerait un avantage économique par rapport à la vaccination active de ces derniers.

La CPS de *S. suis* est une cible intéressante considérant la possibilité de créer un vaccin polyvalent protégeant contre les sérotypes les plus répandus comme c'est le cas chez *S. pneumoniae*. Un tel vaccin pourrait également varier selon la région afin de mieux répondre aux besoins épidémiologiques. Cependant, la CPS seule de *S. suis* est très peu immunogénique. La recherche sur les vaccins basés sur les épitopes capsulaires est présentement axée sur l'obtention d'un vaccin conjugué (CPS-protéine) afin d'augmenter le potentiel immunogénique de la CPS. Récemment, un conjugué CPS-toxine tétanique a été développé avec la CPS du sérotype 2 de *S. suis*. Il a été observé que le conjugué induit des anticorps opsonisants IgM et IgGs et confère une protection lors d'une infection expérimentale avec une dose létale chez le porc[61].

Alors que les vaccins avec des bactéries tuées (« bactérines ») ou atténuées démontrent une efficacité limitée, d'autres cibles utilisées pour des vaccins sont les protéines de surface. Plusieurs protéines ont été testées dans des essais de vaccination; dont Sao, SLY, MRP, EF, et Enolase[59]. Cependant, à ce jour, aucun antigène universel permettant une protection complète contre plusieurs sérotypes n'a été trouvé. Un vaccin sous-unitaire composé de plusieurs différents antigènes de surface pourrait également conférer une protection croisée intéressante.

2. La capsule polysaccharidique (structure, locus et synthèse)

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 29 différents sérotypes capsulaires qui se distinguent par différentes compositions, différentes structures, mais également différentes organisations chromosomiques au sein des loci capsulaires[209]. Plusieurs équipes ont travaillé dans les dernières années à la caractérisation des structures et des loci capsulaires. Dans la prochaine section, les différentes compositions, structures et organisations génétiques de la CPS de différents sérotypes d'intérêt pour cette thèse seront revues.

2.1 Structures et compositions des CPSs

Les structures de la CPS du sérotype 2 (2010), du sérotype 14 (2012), des sérotypes 1 et 1/2 (2015) et du sérotype 9 (2016) chez *S. suis* ont été déterminées par Van Calsteren & al (sérotypes 1, 1/2, 2 et 14) et Vinogradov & al. (sérotype 9)[14, 210, 211]. Nous nous intéresserons plus particulièrement au sérotype 1, 1/2, 2 et 14 dans cette thèse.

2.1.1. Sérotypes 1/2 et 2

La CPS du sérotype 2 est composée de galactose (Gal), glucose (Glu), *N*-acétylglucosamine (GlcNAc), rhamnose (Rha) et de l'acide sialique (Neu5Ac). La composition du sérotype 1/2 est identique à la CPS du sérotype 2, sauf qu'elle contient du *N*-acétylgalactosamine à la place du galactose de la chaîne latérale portant l'acide sialique. La séquence répétitive des sous-unités du sérotype 2 est [4)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)]Gal(β1–4)[Gal(α1–3)]Rha(β1–4)Glc(β1-]$_n$. On peut remarquer la présence de l'acide sialique relié au galactose par un lien spécifique α2-6 (**Figure 4**). Il est proposé que la chaine latérale et l'acide sialique constitue les principaux épitopes reconnus par les anticorps dirigés contre la CPS des sérotypes 1/2 et 2. Malgré les structures similaires des chaines

latérales entre les sérotypes 2 et 14 (voir ici-bas), l'absence de réaction croisée en sérologie démontre l'importance de la conformation structurale de la CPS dans la reconnaissance par les anticorps[14, 210].

Curieusement, malgré des compositions et des structures similaires, il existe une différence au niveau de la longueur des chaînes polysaccharidiques. En effet, chez le sérotype 2, la longueur des chaînes (représentée par la masse moléculaire) est de 414 kg/mol alors que chez le sérotype 1/2, les chaînes sont d'une masse moléculaire de 709 kg/mol[14, 210]. La cause exacte de cette différence n'est pas connue, mais l'efficacité de la polymérase pourrait être impliquée.

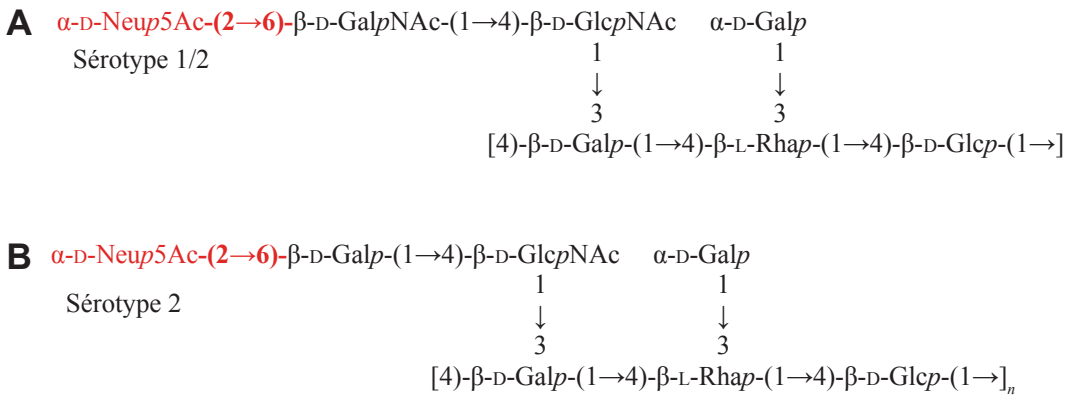


Figure 4. Structures de la CPS polysaccharidique des sérotypes 1/2 (A) et 2 (B). L'acide sialique (α2,6) est représenté en rouge. La seule différence entre les deux structures est la présence d'un N-acétylgalactosamine relié à l'acide sialique chez le sérotype 1/2 à la place d'un galactose chez le sérotype 2. Figure modifiée à partir de Van Calsteren & al. 2010 et 2016[14, 210].

2.1.2. Sérotypes 1 et 14

La CPS du sérotype 14 est composée quant à elle de galactose, glucose, *N*-acétylglucosamine et de l'acide sialique. À l'instar des sérotypes 1/2 et 2, la seule différence entre les CPSs des sérotypes 1 et 14 est la présence du *N*-acétylgalactosamine (GalNAc) chez le sérotype 1 à la place du galactose (Gal(β1-4)). La séquence répétitive des sous-unités du sérotype 14 est [6)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)]Gal(β1-3)Gal(β1-4)Glc(β1-](n). L'acide sialique est également relié au galactose par un lien spécifique α2-6 (**Figure 5**). Chez la CPS du sérotype 1, l'épitope principale semble être également la chaîne latérale. À l'opposé, la chaîne latérale chez le sérotype 14 ne semble pas être un épitope majeur. De plus, l''acide sialique joue un rôle très limité chez les sérotypes 1 et 14 dans la reconnaissance par les anticorps[14, 212]. À l'instar des sérotypes 1/2 et 2, il existe une différence dans la longueur des chaînes polysaccharidiques entre le sérotype 1 (741 kg/mol) et le sérotype 14 (488 kg/mol).

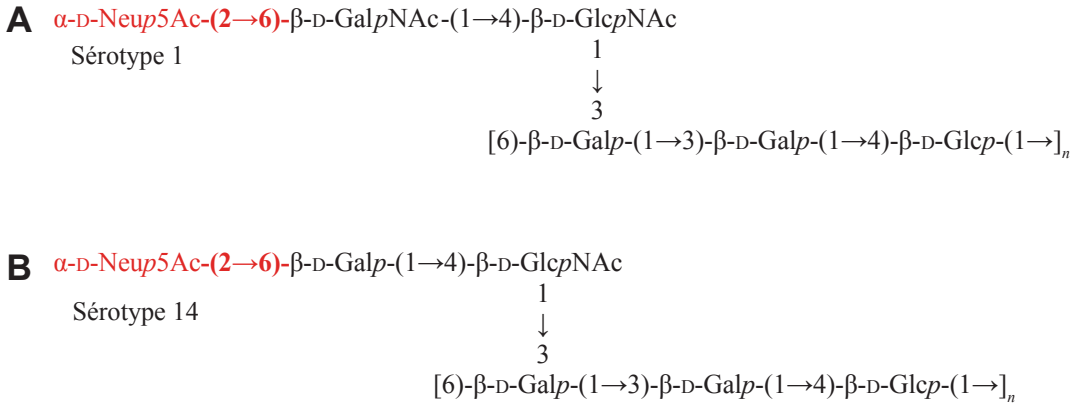


Figure 5. Structures de la CPS polysaccharidique des sérotypes 1 (A) et 14 (B). L'acide sialique (α2,6) est représenté en rouge. La seule différence entre les deux structures est la présence d'un *N*-acétylgalactosamine relié à l'acide sialique chez le sérotype 1 à la place d'un galactose chez le sérotype 14. Figure modifiée à partir de Van Calsteren & al. 2013 et 2016[14, 212].

2.2. Loci capsulaires

Le locus de la CPS de *S. suis* a été caractérisé en premier chez le sérotype 2 par Smith & al. en 1999[49]. Il a fallu cependant attendre en 2013 afin que l'ensemble des différents loci capsulaires correspondant aux 35 sérotypes (maintenant 29) de *S. suis* soit caractérisés par Okura & al.[209]. C'est d'ailleurs sur cette dernière étude publiée par Okura & al. que la nomenclature pour tous les gènes des différents sérotypes a pu être établie. Nous utiliserons cette nomenclature dans ce document.

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 ARN polycistronique et régulé sous un seul même promoteur situé en amont du gène *cpsA*. La taille des loci de la CPS varie entre 15274 et 40198 paires de bases en fonction du sérotype. Les gènes codant pour la synthèse, l'exportation et la polymérisation de la CPS se retrouvent chez la plupart des sérotypes entre les gènes *orfZ* et *aroA*. Néanmoins, chez 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 loci chromosomiques ont pu être établis auprès des 35 sérotypes, soit les patrons Ia, Ib, II, III et IV. Le patron le plus commun est le patron Ia comprenant 22 sérotypes notamment les sérotypes 1, 1/2, 2 et 14. Le second groupe plus représenté est le patron Ib, compris entre *orfZ* et *glf*, qui comprend les sérotypes 9, 13, 21, 24, 29, 31 et 34[209].

Le locus de la CPS varie entre les différents patrons chromosomiques de loci capsulaires, par contre il existe certaines caractéristiques communes chez plusieurs sérotypes. Ainsi, on retrouve les gènes de régulation de la synthèse de la CPS, *cpsA*, *cpsB*, *cpsC* et *cpsD* chez tous les sérotypes. Les gènes codant pour des polymérases, flippases et différentes glycosyltransférases sont également retrouvés chez l'ensemble des sérotypes. Une caractéristique importante est la présence chez certains sérotypes de gènes associés à la

synthèse et au transfert (sialyltransférase) de l'acide sialique. Ces gènes, soit *neuBCDA* (synthèse) et *cpsN* (sialyltransférase) ont été caractérisés chez les sérotypes 1 (**Tableau II**), 1/2 et 2 (**Tableau III**), 6, 13, 14, 16 et 27. Curieusement, les gènes codant pour la synthèse de l'acide sialique chez le sérotype 13 sont phylogénétiquement différents des autres sérotypes sialylés mentionnés[209].

Tableau II. Fonctions des gènes du locus capsulaire du sérotype 1

Gènes	Fonctions
cps1A	Protéine régulatrice intégrale membranaire
cps1B	Protéine régulatrice (longueur chaîne et exportation)
cps1C	Tyrosine-protéine kinase (Wze)
cps1D	Protéine-tyrosine phosphatase (Wzh)
cps1E	Transférase initiale (transférase glucosyl-1-phosphate)
cps1F	Glycosyltransférase
cps1G	Glycosyltransférase (galactosyltransférase potentielle)
cps1H	Glycosyltransférase (galactosyltransférase potentielle)
cps1I	Polymérase (Wzy)
cps1J	Glycosyltransférase
cps1K	Glycosyltransferase (acétylglucosaminyltransférase potentielle)
cps1L[1]	Glycosyltransférase (galactosyltransférase potentielle)
cps1M	Protéine potentielle
cps1N	Protéine potentielle
cps1O	Sialyltransférase
cps1P	Flippase (Wzx)
neu1B	Acide *N*-acétylneuraminique synthase
neu1C	UDP-*N*-acétylglucosamine 2-épimérase
neu1D	Acide *N*-acétylneuraminique synthase (O-acétyltransférase potentielle)
neu1A	*N*-acétylneuraminate cytidylyltransférase

[1] Gène homologue à *cps14K, cps2K* et *cps1/2K*

Outre les gènes mentionnés précédemment, les loci capsulaires comprennent également un gène codant pour la transférase initiale, identifié *cpsE* chez les sérotypes 1, 1/2, 2, 6, 8, 14, 15, 16, 20, 22, 25, 27, 32, 34[209].

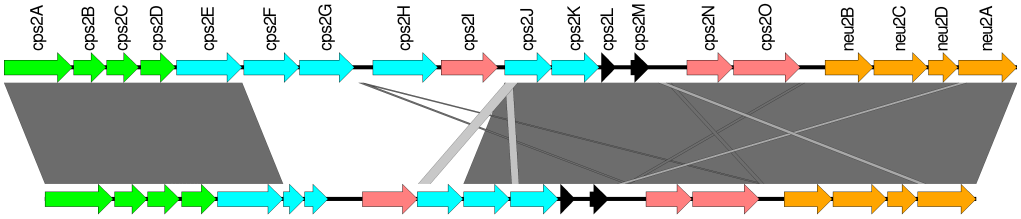
Tableau III. Fonctions des gènes des loci capsulaires des sérotypes 1/2 et 2

Gènes[1]	Fonctions
cps1/2A* et *cps2A	Protéine régulatrice intégrale membranaire
cps1/2B* et *cps2B	Protéine régulatrice (longueur chaîne et exportation)
cps1/2C* et *cps2C	Tyrosine-protéine kinase (Wze)
cps1/2D* et *cps2D	Protéine-tyrosine phosphatase (Wzh)
cps1/2E* et *cps2E	Transférase initiale (transférase glucosyl-1-phosphate)
cps1/2F* et *cps2F	Glycosyltransférase (rhamnosyltransférase potentielle)
cps1/2G* et *cps2G	Glycosyltransférase (galactosyltransférase potentielle)
cps1/2H* et *cps2H	Glycosyltransférase (galactosyltransférase potentielle)
cps1/2I* et *cps2I*	Polymérase (Wzy)
cps1/2J* et *cps2J*	Glycosyltransférase (acétylglucosaminyltransférase potentielle)
cps1/2K* et *cps2K	Glycosyltransférase (galactosyltransférase potentielle)
cps1/2L* et *cps2L	Protéine potentielle
cps1/2M* et *cps2M	Protéine potentielle
cps1/2N* et *cps2N*	Sialyltransférase
cps1/2O* et *cps2O*	Flippase (Wzx)
neu1/2B* et *neu2B	Acide *N*-acétylneuraminique synthase
neu1/2C* et *neu2C	UDP-*N*-acétylglucosamine 2-épimérase
neu1/2D* et *neu2D	Acide *N*-acétylneuraminique synthase (O-acétyltransférase potentielle)
neu1/2A* et *neu2A	*N*-acétylneuraminate cytidylyltransférase

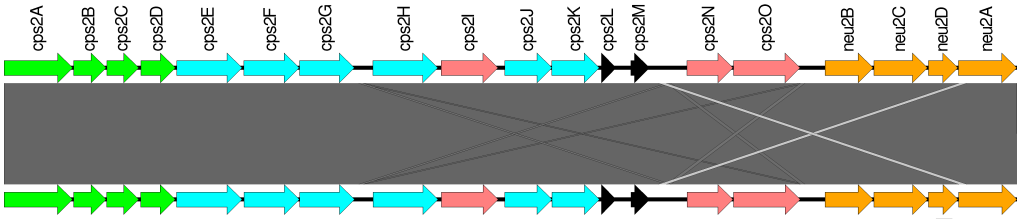
[1] Les gènes en rouges représentent les gènes mutés et caractérisés dans la littérature (phénotype non encapsulé)
* Ces gènes ont été caractérisés comme létales chez *S. suis* et des mutants de ces gènes sont possibles uniquement en présence de mutations suppressives (voir section 3.6.3. Mutagenèse des gènes de la CPS et létalité chez les streptocoques pathogènes)

La plupart des sérotypes possèdent un gène sérotype-spécifique. Les exceptions sont les sérotypes 1, 1/2, 2 et 14 où aucune polymérase, glycosyltransférase ou flippase sérotype-spécifique n'a été trouvée. Une particularité est la grande similitude entre les locus des sérotypes 1, 1/2, 2 et 14. De plus, les locus de la CPS des sérotypes 1 et 14 et des sérotypes 2 et 1/2 sont pratiquement identiques, d'où l'absence de gène sérotype-spécifique (**Figure 6**). Une différence importante dans les loci de la CPS entre les sérotypes 2 et 14 et les sérotypes 1 et 1/2, est la présence d'une rhamnosyltransférase (*cps2F*) chez les sérotypes 1/2 et 2 (**Tableau II, III et IV**). Curieusement, il existe une version tronquée non fonctionnelle de ce gène chez les sérotypes 1 et 14. En effet, la détermination de la structure de la CPS du sérotype 14 et du sérotype 1 a permis de démontrer que le rhamnose était bien absent de la CPS chez les sérotypes 1 et 14[209, 212].

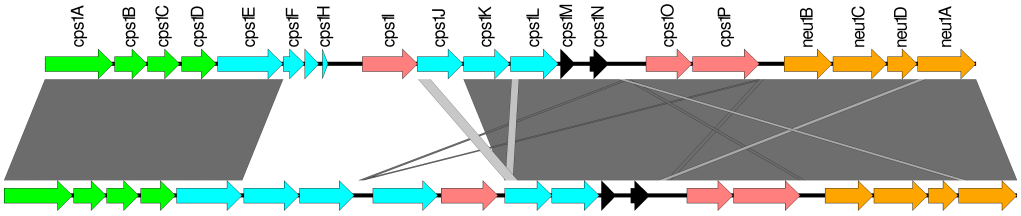
A. Alignement des loci capsulaires des sérotypes 2 et 14



B. Alignement des loci capsulaires des sérotypes 2 et 1/2



C. Alignement des loci capsulaires des sérotypes 1 et 2



D. Alignement des loci capsulaires des sérotypes 1 et 14

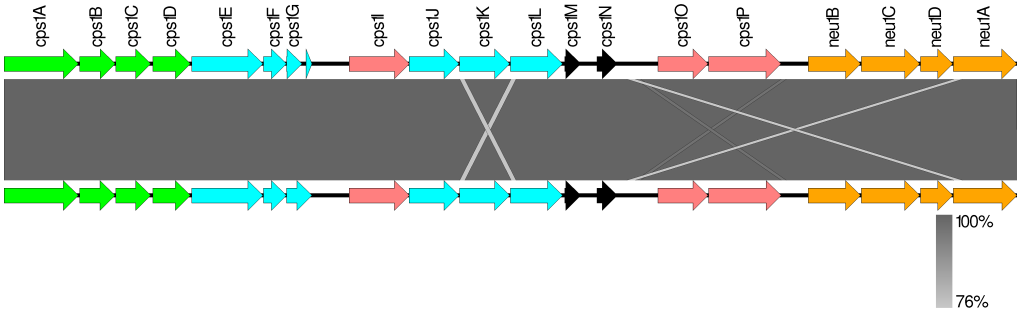


Figure 6. Comparaison génétique des loci capsulaires des sérotypes 1, 1/2, 2 et 14. (A) Comparaison des loci capsulaires des sérotypes 2 et 14, (B) Comparaison des loci capsulaires des sérotypes 2 et 1/2, (C) Comparaison des loci capsulaires des sérotypes 1 et 2 et (D) Comparaison des loci capsulaires des sérotypes 1 et 14. Le pourcentage d'homologie entre les séquences nucléiques est illustré par les tons de gris, où le plus foncé démontre une très forte homologie et les zones claires, une faible homologie. Couleurs: vert; gènes de régulation, cyan; glycosyltransférases, rouge; enzymes clés

(polymérase, sialyltransférase et flippase) et orange; gènes responsables de la synthèse de l'acide sialique.

Tableau IV. Fonctions des gènes du locus capsulaire du sérotype 14

Gènes [1]	Fonctions
cps14A	Protéine régulatrice intégrale membranaire
cps14B	Protéine régulatrice (longueur chaîne et exportation)
cps14C	Tyrosine-protéine kinase (Wze)
cps14D	Protéine-tyrosine phosphatase (Wzh)
cps14E	Transférase (transférase glucosyl-1-phosphate)
cps14F	Glycosyltransférase (rhamnosyltransférase potentielle)
cps14G	Glycosyltransférase (galactosyltransférase potentielle)
cps14H	Glycosyltransférase (galactosyltransférase potentielle)
cps14I	Polymérase (Wzy)
cps14J	Glycosyltransferase (acétylglucosaminyltransférase potentielle)
cps14K	Glycosyltransférase (galactosyltransférase potentielle)
cps14L	Protéine potentielle
cps14M	Protéine potentielle
cps14N	Sialyltransférase
cps14O	Flippase (Wzx)
neu14B	Acide *N*-acétylneuraminique synthase
neu14C	UDP-*N*-acétylglucosamine 2-épimérase
neu14D	Acide *N*-acétylneuraminique synthase (O-acétyltransférase potentielle)
neu14A	*N*-acétylneuraminate cytidylyltransférase

2.2.2. Groupe d'homologie ou « Homology Groups » (HGs)

Les gènes codant pour la synthèse de la CPS chez les différentes souches peuvent être regroupés en « homology groups » (HG) selon leur similitude de fonction. Le module utilisé, le « pan-genome analysis pipeline » est une plate-forme d'analyse de génomes permettant de regrouper des gènes d'un locus cible ayant des fonctions homologues, ici le locus capsulaire. Chez les 35 sérotypes connues, l'ensemble des gènes codant pour la synthèse de la CPS a été regroupé en 361 HGs. À titre d'exemple, les gènes de régulation conservés chez tous les sérotypes, *cpsA, cpsB, cpsC* et *cpsD* codent pour des fonctions conservées chez tous les sérotypes et conséquemment, représentent chacun un seul HG (HG1-4). À l'opposé, les gènes codant pour la transférase initiale varient en fonction de la composition des chaînes polysaccharidiques et sont regroupés en 7 différents HGs[209].

2.2.3. Polymorphisme nucléotidique (SNP)

Récemment, avec la disponibilité de plusieurs génomes séquencés, des analyses *in silico* des loci capsulaires de plusieurs souches de sérotypes 1, 1/2, 2 et 14 ont permis de cibler un seul nucléotide au sein du gène *cpsK*, qui serait responsable de la spécificité du sucre transféré entre les sérotypes 1 et 14 et les sérotypes 1/2 et 2. Il existe un seul polymorphisme nucléotidique non synonyme (SNP) entre les gènes *cps1/2K* et *cps2K* et entre les gènes *cps1K* et *cps14K*. Il est proposé que le polymorphisme de l'acide nucléique 483 du gène *cpsK* permet de coder soit pour une *N*-acétylgalactosamine (T483G) chez les sérotypes 1 et 1/2 où soit pour une galactosyltransférase (G483T) pour les sérotypes 2 et 14[213].

Tel que démontré par Van Calsteren & al.[14] les chaînes latérales semblent être des épitopes majeures chez ces sérotypes, à l'exception du sérotype 14, et seraient responsables des réactions croisées observées[14]. Néanmoins, la responsabilité du SNP dans le gène *cpsK* dans les différences d'antigénicités observées entre les sérotypes 1, 1/2, 2 et 14 reste à être démontrée.

2.2.4. Nouveaux loci capsulaires (NCLs) et nouveaux sérotypes potentiels

Récemment, Zhenghan & al.[15] ont décrit les loci capsulaires de 179 souches 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 loci 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 à cause de mutations dans le locus capsulaire, rendant ainsi impossible le sérotypage de ces souches par de futurs tests sérologiques[15].

Parallèlement, Pan & al.[214] 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[214].

Au moment d'écrire cette thèse, huit autres nouveaux loci capsulaires potentiels ont été caractérisés chez *S. suis,* portant le nombre de NCLs à 17 (1-16 et Chz). À l'opposé des premiers NCLs (1-8), ces derniers NCL ont été caractérisés à partir de souches de *S. suis* isolées des porcs sains majoritairement mains également des 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[215]. 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*.

2.2.5. Mutants de la CPS chez S. suis

Le premier mutant non encapsulé chez *S. suis* a été obtenus en 1998 par insertion de transposon. Il a été démontré qu'une seule insertion par transposon était suffisante afin d'inhiber la synthèse de la CPS chez *S. suis*. Malgré que le site exact de l'insertion du transposon n'est pas décrit dans ces travaux, ce mutant non encapsulé de *S. suis* a permis de démontrer pour la première fois que l'absence de CPS modifie l'hydrophobicité à la surface de la bactérie, diminue la résistance à la phagocytose par les phagocytes d'origine murins et porcins et favorise l'élimination rapide de la bactérie du sang[216].

Au moment d'amorcer mon projet de doctorat, les rôles de la protéine régulatrice CpsB, de la transférase initiale CpsE, de la rhamnosyltransférase (CpsF) ainsi que de la UDP-*N*-acétylglucosamine 2-épimérase (NeuC; enzyme impliqué dans la synthèse de l'acide sialique) ont été décrits à l'aide de mutants déficients en la synthèse de ces protéines chez le sérotype 2 uniquement[217, 218].

Curieusement, tous les mutants déficients en ces quatre protéines, CpsB, CpsE/F et NeuC démontrent le même phénotype, soit un phénotype non encapsulé. Chez le sérotype 2, il semble qu'une mutation dans un gène régulateur (*cpsB*), une glycosyltransférase (*cpsE/F*) ainsi que dans un gène de la voie de synthèse de l'acide sialique (*neuC*), inhibe complètement la production de la CPS. Au début de thèse, aucun mutant déficient dans l'expression d'un gène codant pour une polymérase, flippase ou sialyltransférase n'avait encore été obtenu et caractérisé. De plus, aucune étude n'avait évalué l'impact d'une délétion dans les gènes codant pour la synthèse de la CPS chez d'autres sérotypes.

2.3. Synthèse 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, nommé en fonction du mécanisme d'exportation de la CPS, existe uniquement chez les bactéries à Gram négatif. Les bactéries à Gram positif utilisent quant à eux 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*). Alors que la voie de la synthase utilise qu'une seule enzyme, la synthase, afin de synthétiser et d'exporter la CPS. En règle générale, une espèce bactérienne va utiliser l'une ou l'autre des voies de synthèse de la CPS. Une exception intéressante est chez *S. pneumoniae*, où la majorité des sérotypes utilisent la voie *wzy*, alors que certains d'autres, notamment le sérotype 3, utilisent la voie de la synthase[219]. Basé sur la présence de gènes codant les différentes enzymes des loci capsulaires des différents sérotypes, c'est-à-dire les

glycosyltransférases, les polymérases et les flippases, il est accepté que la synthèse de la CPS de *S. suis* se fait via la voie de la polymérase *(wzy)*[209].

2.3.1. La voie wzy

La voie wzy peut se traduire en 4 étapes; (i) l'initiation, (ii) la formation des unités répétées, (iii) l'exportation et (iv), la polymérisation et finalement (v) la translocation au peptidoglycane **(Figure 7)**[219].

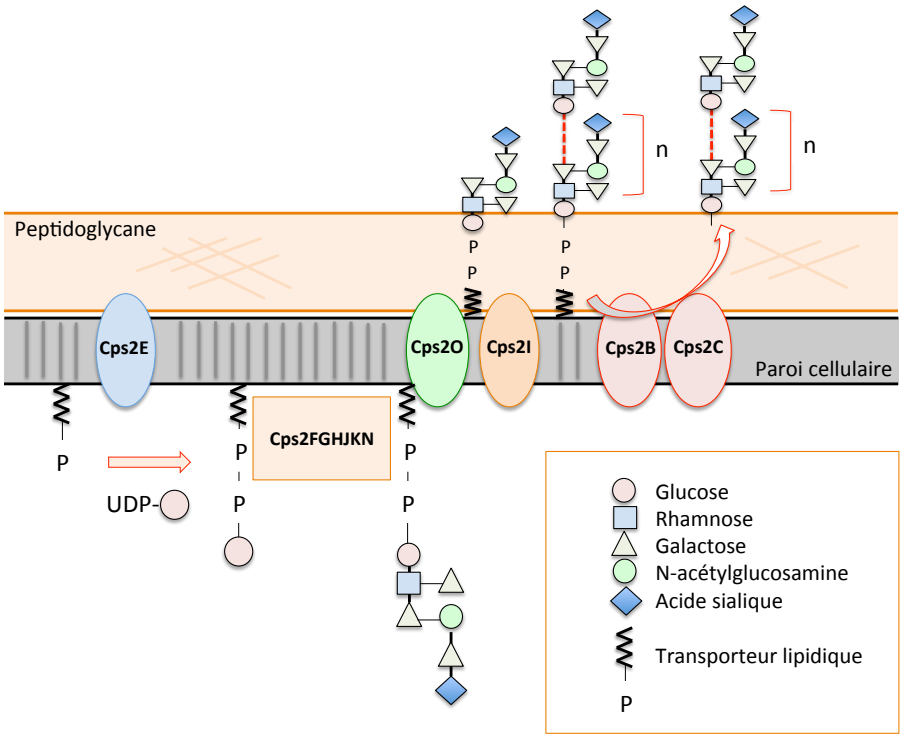


Figure 7. Synthèse de la CPS polysaccharidique de *S. suis* sérotype 2 par la voie de synthèse 'wzy'. Les deux enzymes clés sont la polymérase Wzy (Cps2I) et la flippase Wzx (Cps2O). L'initiation de la synthèse de la CPS débute avec le transfert d'un sucre-1-phosphate au précurseur lipidique (Und-P), le même accepteur qui est utilisé dans la synthèse du peptidoglycane et l'acide téichoïque chez les bactéries à Gram positif. Après l'initiation, des 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. La polymérase effectue la polymérisation en chaînes polysaccharidiques. Le complexe Wzd/Wze (codé par *cpsB*/*cpsC*) transfert ensuite la CPS mature 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. Figure inspirée de Lakkitjaroan & al. 2014[220].

(**i**) La synthèse de la CPS commence par le transfert d'un sucre-1-phosphate à un transporteur lipidique, le lipide 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 synthèse du peptidoglycane et de l'acide téichoïque. La voie de synthèse 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, dont les sérotypes 1, 1/2, 2 et 14[219].

(**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. On y retrouve notamment des galactosyltransférases, des rhamnosyltransférases, des acétylglucosaminyltransférases et l'enzyme permettant la sialylation des sous-unités polysaccharidiques, l'acide *N*-acétylneuraminique transférase (sialyltransférase). La nature des glycosyltransférases varie en fonction du sérotype et dicte ainsi la composition et la structure de la CPS[219].

(**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 saccharidique précise[219].

(**v**) Le complexe Wzd/Wze (codé par *cpsB*/*cpsC*) transfert ensuite la CPS mature 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[220].

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)[209].

2.4. Régulation de la CPS

Il a été proposé que *S. suis* module la production de sa CPS en fonction de l'environnement où il se retrouve. Une épaisseur diminuée de CPS favoriserait l'interaction des différentes adhésines avec leurs ligands et augmenterait l'adhésion aux cellules épithéliales de l'hôte, alors qu'une synthèse optimale de la CPS confère à *S. suis* des propriétés antiphagocytaires lui permettant de s'évader des cellules immunitaires lorsqu'il se retrouve dans la circulation sanguine. Cependant, la régulation de la CPS de *S. suis in vivo* reste encore à être démontré.

Néanmoins, certains régulateurs ou protéines sont capables d'influencer l'expression des gènes de la synthèse de la CPS *in vitro*; (i) la protéine régulatrice du métabolisme du carbone (Ccpa), (ii) le régulateur CovR, (iii) le régulateur global CodY et (iv) la di-adénosine monophosphate (AMP) phosphodiestérase. De plus, au moment de finaliser cette thèse, une étude mettant en lumière le rôle d'un ARN messager dans la modulation de la synthèse de la CPS a été publiée[136].

2.4.1. Catabolite Control Protein A (Ccpa)

Les bactéries utilisent un système de contrôle du métabolisme associé au carbone qui permet l'activation ou l'inhibition de l'expression de certains gènes en réponse à la disponibilité du carbone dans le milieu. Ce système appelé « carbon catabolite repression » (CCR), est composé du cofacteur Hpr, d'un système phosphotransférase (PTS) et du régulateur transcriptionnel Ccpa. Lorsque les bactéries sont dans un milieu activant CCR, la protéine kinase du système PTS phosphorylera le cofacteur Hpr au niveau de la sérine 46 (Hpr-(Ser-46-

P)) qui permettra à celle-ci de former un complexe avec Ccpa. Ce complexe, Hpr-(Ser-46-P)-Ccpa, pourra lier les « conserved catabolite responsive elements » (CRE) dans la région des promoteurs de certains gènes et ainsi moduler la transcription de ces gènes[221, 222]. Curieusement, une récente étude a révélé la présence d'un élèment CRE dans l'opéron (*cps2A*) de la capsule de *S. suis* [72]. Outre son rôle dans le système CCR, Ccpa serait également capable, indépendamment du CCR, de moduler certains gènes de virulence, des gènes nécessaires à la sporulation et des gènes de résistance aux antibiotiques[223].

Le rôle de Ccpa dans la régulation de gènes en fonction de la disponibilité des hydrates de carbone a été étudié chez *S. suis*. La suppression du gène de Ccpa chez une souche de *S. suis* en condition de milieu riche en glucose, entraîne une diminution significative de la transcription des gènes du locus de la CPS. Il a également été démontré par microscopie électronique que cette diminution de l'expression des gènes de la CPS entraînait une diminution de la quantité de CPS exprimée à la surface de la bactérie. Outre les gènes de la CPS, d'autres gènes codant pour des facteurs de virulence (*sao, ofs, eno*) sont également réprimés chez le mutant déficient en la production de Ccpa. Néanmoins, malgré la réduction marquée de l'épaisseur de la CPS, aucune différence dans l'adhésion aux cellules épithéliales et dans la formation de biofilm entre le mutant Δ*ccpa* et la souche mère n'a été observé[126].

2.4.2. Systèmes à deux composants (TCSs); CovR et SalK/SalR

Outre la disponibilité des hydrates de carbone, les bactéries possèdent des systèmes leur permettant de répondre à divers stimuli. Les systèmes à deux composants (TCS) sont des systèmes composés d'une protéine bifonctionnelle senseur/histidine kinase (CovS) et un effecteur (CovR), généralement une protéine pouvant lier l'ADN et en moduler la transcription. Lorsque la protéine histidine-kinase perçoit un changement dans le milieu, elle s'autophosphoryle, permettant le transfert d'un groupement phosphate à l'effecteur (CovR). Cette phosphorylation de l'effecteur influence son affinité de liaison pour ces promoteurs cibles, et conséquemment module la transcription de ces gènes[129].

Le système à deux composants CovS (senseur) / CovR (effecteur) est impliqué dans la régulation de gènes de virulence de plusieurs streptocoques, notamment chez *S. pyogenes* et GBS. Une particularité chez *S. suis* est l'absence du gène codant pour CovS dans le génome. Il est suggéré qu'une protéine kinase alternative agit comme donneuse du groupement phosphate[129].

Étonnamment, le mutant Δ*covR* de *S. suis* adhère davantage aux cellules (épithéliales et endothéliales), résiste davantage à la phagocytose et à la destruction (par les neutrophiles et monocytes) et possède une virulence augmentée par rapport à la souche mère. Le manque de régulation des différents gènes de virulence chez le mutant Δ*covR* mène à une surexpression de ceux-ci s'avérant être avantageux pour la virulence du pathogène (mutant Δ*covR*) dans un modèle *in vitro,* mais probablement au détriment de certaines fonctions métaboliques. La fine régulation des facteurs de virulence est un concept clé en pathogenèse et permet à la bactérie d'utiliser optimalement l'énergie dont elle dispose[129].

Le gène de la CPS *cps2C* est l'un des gènes surexprimés chez le mutant. Il a été démontré par microscopie électronique que le mutant exprimait une CPS plus épaisse, d'où la plus grande résistance à la phagocytose et à l'élimination par les monocytes et neutrophiles observée dans cette étude [129]. Cependant, ce résultat contredit l'adhérence plus marquée du mutant aux cellules épithéliales et endothéliales, étant donné que la présence de la CPS influence négativement l'adhérence aux cellules chez *S. suis*.

Outre CovR, le système à deux composants SalK/SalR a également été décrit comme potentiel régulateur de la CPS. Le mutant déficient en le système SalK/SalR de la souche 05ZYH33 démontre un phénotype non encapsulé et une grande susceptibilité à la phagocytose[224].

2.4.3. CodY

La protéine CodY est une protéine liant le GTP qui a été caractérisé pour la première fois pour son rôle dans l'initialisation de la sporulation chez *Bacillus subtilis*. La protéine CodY est retrouvée dans pratiquement toutes les bactéries à Gram positif de faible contenu en G+C et permet la régulation de l'expression de gènes nécessaires à l'adaptation dans les milieux pauvres. La liaison avec la GTP active les propriétés répressives de CodY. La protéine CodY est capable de lier directement l'ADN afin de réprimer la transcription de certains gènes. La faible affinité de CodY pour la GTP lui permet de détecter les faibles changements dans le milieu de croissance et de réguler ces gènes en fonction de la disponibilité des nutriments[225].

La mutation de la protéine CodY chez *S. suis* influence la synthèse de la CPS. Feng & al.[128] ont démontré à l'aide d'un mutant déficient en la protéine CodY chez *S. suis*, que la CPS était significativement diminuée par rapport à la souche mère durant la phase exponentielle et stationnaire. Une analyse de l'expression des gènes a permis de démontrer que le gène codant pour une glycosyltransférase *cps2J* ainsi que les gènes associés à l'acide sialique *neuB, neuC* et *neuD* étaient régulés à la baisse chez le mutant. Outre la diminution de la CPS, ils ont pu observer une diminution de l'adhésion et de l'invasion des cellules épithéliales Hep-2 et une augmentation de la résistance à la phagocytose chez le mutant par rapport à la souche mère[128]. Encore une fois, ces observations sont en contradiction avec le fait qu'une souche moins encapsulée devrait être plus adhérente et moins résistante à la phagocytose. Ainsi, les rôles des systèmes à deux composants et du régulateur CodY restent à être confirmés.

2.4.4. Le second messager nucléotidique di-AMP cyclique (di-AMPc)

Le second messager di-AMPc est également impliqué dans la régulation de la CPS. Le système de régulation via le second messager di-AMPc est très peu caractérisé chez *S. suis,* mais il est retrouvé de manière quasi ubiquitaire chez les bactéries. Ce système permet, en autre, de s'adapter à l'environnement via des stimuli externes, régule la formation de biofilm et la virulence de plusieurs pathogènes[226, 227]. Le niveau cellulaire du di-AMPc varie en fonction

de sa dégradation et de sa synthèse via des protéines phosphodiestérase (dégradation) et diadénylate cyclases (synthèse). Le di-AMPc est capable de lier directement la protéine cible ou l'ARN cible afin de moduler leurs fonctions.

Chez *S. suis*, il a été démontré que le di-AMPc est capable d'influencer la transcription des gènes du locus de la CPS. Cependant, l'impact phénotypique de la production de la CPS à la surface de *S. suis* n'a pas été caractérisé. Le mutant de di-AMPc chez *S. suis* a démontré une diminution de l'adhésion et de l'invasion des cellules épithéliales Hep-2 et une diminution de la virulence dans un modèle d'infection murin[69]. Le rôle de la CPS dans ce phénotype reste à être confirmé.

2.4.5. Le petit ARN rss04 (small RNA rss04)

Le petit ARN ou *small RNA* (ARNs) rss04 est également impliqué dans la régulation de la CPS. Malgré que les mécanismes de régulation exercés par l'ARN messager *rss04* ne sont pas connus, il a été démontré que l'ARNs *rss04* réprime l'expression du régulateur transcriptionnel *ccpa*. Une fois réprimé, le régulateur Ccpa serait incapable de contrôler la synthèse de la CPS. Chez le mutant déficient en l'expression de *rss04*, la production de Ccpa et l'épaisseur de la CPS sont significativement augmentées par rapport à la souche mère, ce qui suggère que *rss04* réprime (contrôle) la synthèse de la CPS, via Ccpa chez la souche mère. De plus, les auteurs ont observé une diminution de l'adhésion et de l'invasion aux cellules endothéliales (BMEC) chez le mutant Δ*rss04*, suggérant un rôle de *rss04* dans la modulation de la synthèse de la CPS au site du SNC afin de favoriser l'établissement de la méningite. Néanmoins, les résultats d'adhésion et d'invasion présentés dans cet article sont contradictoires, étant donné qu'une augmentation de la CPS interfère avec l'adhésion de *S. suis* aux cellules de l'hôte. La diminution de la production de la CPS à la surface de la bactérie permettrait de faciliter l'interaction entre les protéines de surface et leurs ligands et ainsi augmenter l'adhésion[136].

Outre la modulation de l'adhésion, la capacité à former des biofilms est réprimée chez le mutant Δ*rss04*. La virulence du mutant Δ*rss04* est également atténuée par rapport à la souche mère. Néanmoins, plusieurs autres facteurs de virulence (91), dont LuxS, sont influencés par l'absence de l'expression de *rss04* chez le mutant Δ*rss04*. Ces facteurs sont également susceptibles d'influencer l'adhésion et la virulence du mutant. Le petit ARN *rss04* pourrait être un facteur important dans la pathogenèse de la méningite causée par *S. suis* étant donné son rôle important dans la modulation de la synthèse de la CPS et, conséquemment, de l'adhésion aux cellules endothéliales[136].

2.5. La CPS polysaccharidique du streptocoque du groupe B

Le streptocoque du groupe B (GBS), nommé selon la classification de Lancefield, est une bactérie commensale de la flore vaginale et représente la cause la plus commune d'infection néonatale. GBS peut causer la méningite, septicémie, pneumonie et arthrite. La CPS de GBS est également l'unité à la base du sérotypage et permet la distinction de dix types différents (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX)[228]. Nous nous intéresserons davantage aux types III et V, qui sont parmi les types les plus fréquemment isolés (avec le type Ia) dans les cas de méningites et de décès néonatal chez l'humain[229]. Malgré la similarité de structure et de composition entre plusieurs sérotypes, notamment entre le type Ia et III, aucune réaction croisée n'est reportée chez GBS[230, 231].

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 CPSs 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)[232]. Étant donné la présence de l'acide sialique dans tous les types de CPS chez GBS, il est avancé que l'acide sialique (α2,3) est un élément clé dans l'évasion des défenses immunitaires de l'hôte[231].

Malgré la similarité de composition et de structure avec *S. suis*, la CPS de GBS ne semble pas être un facteur antiphagocytaire aussi important que chez *S. suis*. En effet, il a été démontré que GBS type III est facilement phagocyté par les BmDCs et les macrophages. GBS se caractérise par une grande survie intracellulaire 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[233, 234].

2.5.1. Locus génétique de la CPS de GBS type III et type V

L'organisation et la composition génique des loci des CPSs de GBS, notamment les types III et V, sont très similaires à *S. suis* (**Figure 8**). Les loci de GBS comportent les gènes de régulation *cpsABCD*, une transférase initiale, diverses glycosyltransférases, une polymérase, une flippase et les gènes codant pour la synthèse et le transfert de l'acide sialique, c'est-à-dire les gènes *neuBCDA* et la sialyltransférase (*cpsK*), respectivement. Curieusement, les CPSs des types II et VIII ont des chaînes polysaccharidiques uniques et possèdent également des sialyltransférases significativement différentes des sialyltransférases des autres types capsulaires. Il est proposé que la spécificité des sialyltransférases de GBS dépendrait non seulement de la reconnaissance du galactose terminale, mais également des autres résidus auxquels le galactose est relié[231, 232]. Une particularité du gène *neuD* (O-acétyltransférase) est qu'un seul polymorphisme nucléotidique (SNP) dans ce gène est associé avec différent phénotype d'O-acétylation chez GBS type III[235].

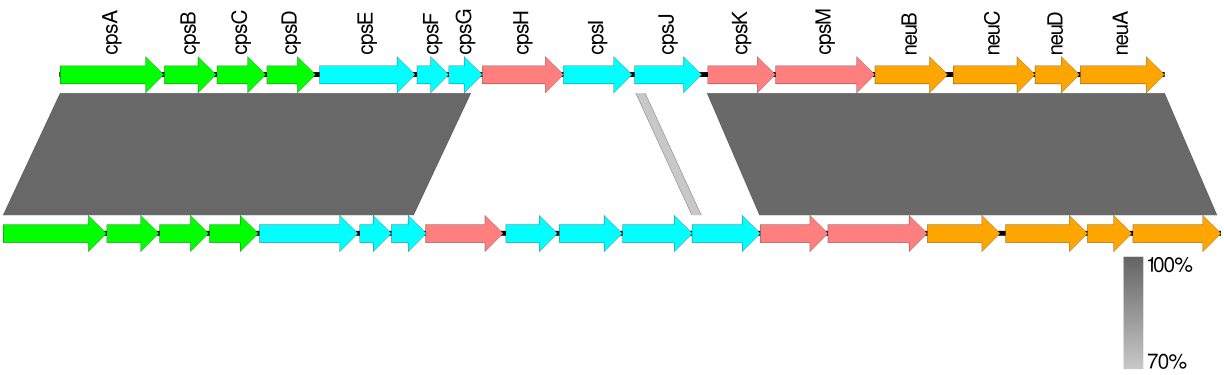
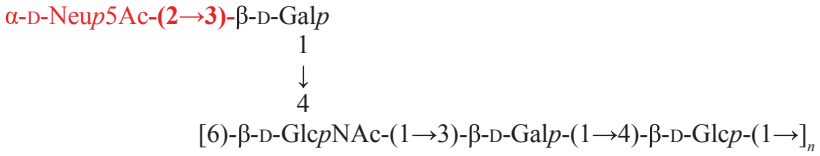


Figure 8. Comparaison génétique des loci capsulaires de GBS type III et V. Le pourcentage d'homologie entre les séquences nucléiques est illustré par les tons de gris, où le plus foncé démontre une très forte homologie et les zones claires, une faible homologie. Des différences significatives sont observées entre le locus du type III (haut) et du type V (bas), notamment au niveau du gène codant pour la polymérase (*cpsH*) et de certaines glycosyltransférases (*cpsI* et *cpsJ*). Couleurs: vert; gènes de régulation, cyan; glycosyltransférases, rouge; enzymes clés (polymérase, sialyltransférase et flippase) et orange; gènes responsables de la synthèse de l'acide sialique.

2.5.2. Structure, composition et fonctionnalité de la CPS de GBS type III

Les chaînes polysaccharidiques des types Ia, Ib, III et V sont toutes composées de galactose, glucose, *N*-acétylglucosamine et d'acide sialique (**Figure 9**). Cependant, la spécificité des liens entre les sous-unités et la structure du squelette en font des CPSs antigéniquement différentes. Une particularité du type III est que la composition et la structure de la CPS sont identiques à celle du pneumocoque du sérotype 14, outre l'absence de l'acide sialique chez *S. pneumoniae*[231]. Malgré cette grande similarité, les anticorps opsonisants naturellement développés contre *S. pneumoniae* sérotype 14 ne sont pas opsonisants pour GBS, suggérant un rôle important de l'acide sialique dans la reconnaissance des anticorps, et donc dans l'épitope immunodominant. De plus, la désialylation de la CPS de GBS type III inhibe la reconnaissance de celle-ci par les anticorps polyclonaux monospécifiques dirigés contre la CPS du type III native, confirmant le rôle de l'acide sialique comme épitope principal chez le type III[236].

A



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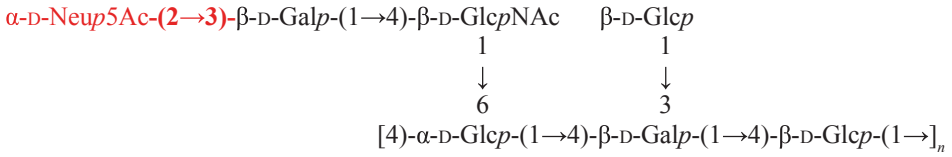

Figure 9. Structures de la CPS polysaccharidique de GBS types III (A) et V (B). L'acide sialique (α2,3) est représenté en rouge. Figure modifiée à partir de Cieslewicz & al 2012[82].

Il existe différents mutants de différents gènes du locus capsulaire qui ont été obtenus et caractérisés chez GBS type III. À titre de comparaison avec *S. suis*, nous discuterons des différences phénotypiques des différents types de mutants capsulaires obtenus chez GBS type III. Au moment de débuter cette thèse, aucun mutant chez GBS type V n'a été caractérisé.

Tout comme chez *S. suis*, des mutants ont été obtenus chez GBS pour les gènes codant les protéines régulatrices (*cpsABCD*)[237], les gènes codant pour des glycosyltransférases[217] ainsi que la plupart des gènes codant pour des protéines impliquées dans la voie de synthèse de l'acide sialique (*neuBCDA*)[217, 238]. De plus, contrairement à *S. suis*, un mutant de la sialyltransférase (*cpsK*) a été obtenu chez GBS type III[232]. Les différents mutants de GBS type III et leur phénotype sont listés dans le **Tableau V**.

Tableau V. Fonctions des gènes du locus capsulaire de GBS type III

Gènes	Fonctions
cps3A	Protéine régulatrice intégrale membranaire
cps3B*	Protéine régulatrice (longueur chaîne et exportation)
cps3C*	Tyrosine-protéine kinase (Wze)
cps3D*	Protéine-tyrosine phosphatase (Wzh)
cps3E	Transférase initiale (transférase glucosyl-1-phosphate)
cps3F	Glycosyltransférase
cps3G	Glycosyltransférase (galactosyltransférase potentielle)
cps3H	Polymérase (Wzy)
cps3I	Glycosyltransférase (acétylglucosaminyltransférase potentielle)
cps3J	Glycosyltransférase (galactosyltransférase potentielle)
cps3K	Sialyltransférase
cps3M	Flippase (Wzx)
neu3B	Acide *N*-acétylneuraminique synthase
neu3C	UDP-*N*-acétylglucosamine 2-épimérase
neu3D	Acide *N*-acétylneuraminique synthase (O-acétyltransférase potentielle)
neu3A	*N*-acétylneuraminate cytidylyltransférase

[1] Les gènes en rouges représentent les gènes mutés et caractérisés dans la littérature (phénotype non encapsulé). Les phénotypes des mutants sont discutés dans la section 'Revue de littérature'
* Mutant obtenu chez le sérotype Ia

Il a été démontré chez GBS type III qu'une mutation dans une glycosyltranférase (*cpsE*) résulte en un phénotype non encapsulé, tout comme *S. suis*[217]. Une mutation dans une glycosyltransférase inhibe la synthèse des sous-unités polysaccharidiques et rend la polymérisation de la CPS impossible. Les gènes de régulation *cpsABCD* sont également nécessaires à la synthèse de la CPS chez GBS. Les mutants des gènes *cpsABCD* produisent des traces ou pas de tout de CPS[237, 239].

Une différence importante avec *S. suis* est la possibilité d'obtenir des mutants encapsulés dénués d'acide sialique. Chez GBS type III, les mutants déficients dans l'expression des gènes codant pour la sialyltransférase (Δ*cpsK*), l'O-acétyltransférase (Δ*neuD*), l'acide sialique synthase (Δ*neuB*) et de la cytidyle-triphosphate (CTP) transférase (Δ*neuA*) sont capables d'exprimer une CPS dépourvue d'acide sialique[232, 235, 238]. Chez le mutant de la sialyltransférase (Δ*cpsK*), il a été observé que l'absence de sialylation des sous-unités polysaccharidique avait un impact important sur la quantité de CPS exprimée. Le mutant Δ*cpsK* chez GBS type III démontre une réduction de 80% de la production de sa CPS par

rapport à la souche mère. Une réduction similaire de la production de la CPS a également été observée chez les mutants déficients dans les enzymes de la voie de synthèse de l'acide sialique, c'est-à-dire *neuBDA*. Un autre impact de l'absence de la sialylation des sous-unités est la longueur des chaînes polysaccharidiques. Des analyses par chromatographie d'exclusion de taille (SEC-MALs) ont permis de démontrer que le mutant Δ*cpsK* de GBS type III exprimait une CPS de masse moléculaire supérieure. Il a ainsi été établi que la souche mutante de la sialyltransférase (Δ*cpsK)* exprimait des chaînes polysaccharidiques d'environs 600 d'unités tétrasaccharidiques répétés, alors que la souche mère en exprime des chaînes polysaccharidiques d'environ 115 unités pentasaccharidiques. Le mutant encapsulé dépourvu d'acide sialique Δ*cpsK* présente donc une CPS diminuée, mais avec de plus longues chaînes polysaccharidiques suggérant une plus grande polymérisation des chaînes polysaccharidiques en absence d'acide sialique[232].

Néanmoins, dans une autre étude, le mutant déficient en l'activation de l'acide sialique (Δ*neuA*), démontre plutôt une diminution de la masse moléculaire de la CPS chez GBS type III[238]. Il semble donc exister des différences dans la synthèse de la CPS lorsque l'acide sialique est absent et lorsque la protéine sialyltransférase est absente. L'accumulation d'acide sialique intracellulaire chez le mutant Δ*cpsK* pourrait ainsi influencer le système de régulation *cpsABCD* impliqué dans la modulation de la longueur des chaînes polysaccharidiques.

Il est proposé chez GBS type III que la diminution de la production de la CPS chez les mutants déficients dans la production ou le transfert de l'acide sialique pourrait être due à une diminution de la formation du précurseur oligosaccharidique ou à une diminution de l'affinité pour la flippase, qui résulterait en une exportation diminuée de la CPS à la surface[232].

2.5.3. Structure, composition et fonctionnalité de la CPS de GBS type V

La CPS du type V est composée de glucose, galactose, *N*-acétylglucosamine et d'acide sialique (**Figure 9**). La CPS du type V possède une structure unique composée d'un squelette de trois saccharides (glucose-galactose-glucose) contenant deux chaînes latérales différentes.

Curieusement, l'élimination de l'acide sialique dans la CPS du type V ne modifie pas la reconnaissance de la CPS du type V par des anticorps polyclonaux monospécifique comme c'est le cas chez le type III[240, 241]. Cette caractéristique suggère que les propriétés immunogéniques ou l'effet conformationnel de l'acide sialique sont peut-être dépendants du type capsulaire. Tout comme les autres types capsulaires de GBS, le type V possède une sialyltransférase significativement différente du type III **(Tableau VI)**[231]. Au moment de débuter cette thèse, aucun mutant de la CPS de GBS type V était disponible afin d'étudier l'impact de la CPS dans la pathogenèse de GBS type V et du rôle de l'acide sialique dans la synthèse de celle-ci.

Tableau VI. Fonctions proposées des gènes du locus capsulaire de GBS type V

Gènes	Fonctions
cps5A	Protéine régulatrice intégrale membranaire
cps5B	Protéine régulatrice (longueur chaîne et exportation)
cps5C	Tyrosine-protéine kinase (Wze)
cps5D	Protéine-tyrosine phosphatase (Wzh)
cps5E	Transférase initiale (transférase glucosyl-1-phosphate)
cps5F	Glycosyltransférase
cps5G	Glycosyltransférase (galactosyltransférase potentielle)
cps5H	Polymérase (Wzy)
cps5M	Glycosyltransférase (acétylglucosaminyltransférase potentielle)
cps5N	Glycosyltransférase (galactosyltransférase potentielle)
cps5O	Glycosyltransférase
cps5J	Glycosyltransférase
cps5K	Sialyltransférase
cps5L	Flippase (Wzx)
neu5B	Acide *N*-acétylneuraminique synthase
neu5C	UDP-*N*-acétylglucosamine 2-épimérase
neu5D	Acide *N*-acétylneuraminique synthase (O-acétyltransférase potentielle)
neu5A	*N*-acétylneuraminate cytidylyltransférase

2.6. Structure et fonctionnalité de la CPS chez les streptocoques

2.6.1. Structure, composition et fonctionnalité

La CPS chez les streptocoques est reconnue comme un facteur antiphagocytaire classique protégeant ces pathogènes contre la phagocytose par les cellules du système immunitaire innée[26]. Cependant, lorsqu'on parcourt la littérature, on observe une grande variation dans les propriétés antiphagocytaires de la CPS. Curieusement, certains pathogènes partagent des CPSs pratiquement identiques, comme GBS type III et *S. pneumoniae* type 14, démontrent des propriétés antiphagocytaires différentes, et suggérant un rôle non négligeable d'autres facteurs antiphagocytaires dans la résistance à la phagocytose. Chez *S. suis*, la surreprésentation de certains sérotypes dans des cas d'infection chez l'humain et le porc, notamment les sérotypes 2, 3 et 9 laisse penser à un rôle important de la structure de la CPS dans le pouvoir pathogène des souches[3].

Chez les streptocoques, la CPS peut être composée de plusieurs sucres différents, mais présente également plusieurs structures différentes médiées par le nombre de chaînes latérales et la variation dans la nature des liens glycosydiques. De plus, on observe la présence de certains sucres particuliers chez certaines bactéries, notamment l'acide hyaluronique chez *S. dysgalactiae* et l'acide sialique chez GBS et *S. suis*.

Chez, *S. pneumoniae*, par exemple, on dénote 90 différents sérotypes basés sur l'antigénicité de la CPS[242]. Le grand nombre de CPSs antigéniquement différentes chez *S. pneumoniae* suggère un impact important de la pression sélective sur l'émergence de nouveaux types capsulaires, tout comme chez *S. suis* (29 vrai sérotypes). La CPS de ce pathogène a d'ailleurs été utilisée dans plusieurs études afin d'étudier l'impact de la composition et de la structure sur les propriétés fonctionnelles de la CPS. Tout comme *S. suis*, chez *S. pneumoniae,* seulement quelques sérotypes particuliers sont responsables de la plupart des infections sévères, suggérant un lien entre la virulence et la composition/structure de la CPS [3, 243].

2.6.2. Effet du sérotype dans les propriétés de la CPS chez les streptocoques

La majorité des études sur les propriétés sérotype-spécifiques de la CPS, particulièrement chez *S. suis*, ont été fait avec des souches génétiquement différentes. Par conséquent, l'impact du bagage génétique sur les différences observées ne peut pas être écarté. Une étude très intéressante sur les propriétés fonctionnelles de la CPS a été effectuée chez *S. pneumoniae* avec des mutants isogéniques exprimant différents types capsulaires. Une approche unique impliquant l'introduction de casettes Janus a rendu possible la substitution du locus entier codant pour la CPS chez *S. pneumoniae*[244]. La première transformation vient remplacer le ou les gènes cibles, ici le locus capsulaire. La souche devient alors non encapsulée et subit une seconde transformation où la casette Janus sera remplacée à son tour par un nouveau locus capsulaire. Le principe de recombinaison homologue permet de transformer la souche directement avec de l'ADN génomique d'une souche codant un locus capsulaire différent et d'en substituer les loci. Les auteurs ont ainsi obtenu des mutants isogéniques exprimant une CPS des sérotypes 6B, 7F, 14 et 19F à partir de la souche TGR4, originalement de sérotype 4[242]. La modification du type capsulaire influence significativement la déposition du complément à la surface de la bactérie et la résistance à l'opsonophagocytose. Les résultats ont également été confirmés chez d'autres souches avec d'autres mutants isogéniques[245].

Un autre résultat intéressant obtenu avec des mutants isogéniques est la différence de déposition du facteur H à la surface de la bactérie entre les mutants de *S. pneumoniae* exprimant une CPS de sérotypes 19A ou 19F[242]. La CPS des sérotypes 19A et 19F possèdent des compositions identiques et diffèrent uniquement que par un lien glycosydique. Ce résultat suggère un rôle non négligeable de la structure de la CPS dans les interactions avec des composants cellulaires de l'hôte, notamment le facteur H. Curieusement, il a été démontré que la différence de déposition du facteur H entre les sérotypes capsulaires était largement dépendant de la présence de la protéine liant le facteur H PspC de *S. pneumoniae*[242]. En l'absence de cette protéine, les différences de déposition de facteur H diminuent, suggérant un rôle conformationnel de la structure de la CPS pour de telles interactions.

Chez GBS, les sérotypes III et Ia expriment pratiquement la même CPS, la seule différence étant au niveau de la liaison entre les chaînes polysaccharidiques exercée par la polymérase spécifique à chaque type capsulaire. Il a été démontré que l'expression de la polymérase *cps3H* chez une souche de sérotype Ia, et *vice versa*, permettait la modification de l'antigénicité de la CPS pour un type III ou Ia, respectivement. Cette expérience démontre qu'il est possible de modifier l'antigénicité de la CPS en modifiant une seule enzyme clé de la voie de synthèse de la CPS. Néanmoins, les auteurs de cette étude ont utilisé un modèle d'expression *in trans* et dépend donc de la présence et de l'expression du plasmide, ce qui limite les utilisations dans des modèles expérimentaux *in vivo*[246].

Au moment de débuter cette thèse, aucune étude à l'aide de mutants isogéniques exprimant des types capsulaires différents n'a été réalisée chez *S. suis*. Cependant, une des limitations à la technique Janus décrite est la difficulté à échanger de longs fragments d'ADN (locus capsulaire). Il est à noter que le locus capsulaire de *S. suis* code pour davantage de gènes (synthèse de l'acide sialique) et est encore plus grand que celui de *S. pneumoniae*. La substitution du locus capsulaire entier chez *S. suis* pourrait s'avérer très difficile ou voir impossible à faire. L'impact réel de la structure et de la composition de la CPS sur la virulence de *S. suis* demeure inconnu.

2.6.3. Mutagenèse des gènes de la CPS et létalité et chez les streptocoques pathogènes

Curieusement, il a été démontré que des mutations dans certaines enzymes clés de la voie de synthèse wzy étaient létales chez certains streptocoques. Chez *S. pneumoniae*, des mutations dans les gènes *cps2K, cps2J* et *cps2H* codant pour la synthèse des chaînes latérales, dans la polymérase et dans la flippase sont létales. Il est proposé que la létalité de ces mutations est causée par la séquestration du précurseur lipidique undécaprényle-phosphate (Und-P) dans la voie de synthèse de la CPS, le rendant indisponible pour les autres fonctions biologiques comme la synthèse du peptidoglycane. Une seconde hypothèse est que l'accumulation du précurseur lipidique déstabiliserait la membrane et nuirait à la réplication de la bactérie[247].

Chez *S. suis*, la létalité de certaines mutations a également été caractérisée. Il a été observé qu'une mutation dans les gènes codant pour les enzymes formant les chaînes latérales *cps2J* (une glycosyltransférase) et *cps2K* (la sialyltransférase), la polymérase et la flippase est létale. Néanmoins, l'obtention de mutants de ces gènes est possible en présence d'une mutation suppressive inhibant la synthèse de la CPS. Les observations sur les souches mutantes obtenues ont déterminé que dans chacun des cas, une mutation dans un autre gène nécessaire à la synthèse de la CPS était présente. Il a été observé que la majorité des mutations suppressives se retrouvent dans la transférase initiale codée par le gène *cpsE*, suggérant un rôle central de cette protéine dans la synthèse de la CPS[220].

Néanmoins, la présence de mutations dans d'autres gènes que *cpsE/F (11/32)*, démontre que les mutations suppressives ne sont pas exclusives a *cpsE/F* et peut arriver dans d'autres gènes nécessaires à la synthèse de la CPS, notamment dans les gènes codant pour les autres glycosyltransférases et les gènes codant pour la synthèse d'acide sialique.

3. Acide sialique

3.1. L'acide sialique chez les pathogènes

Le terme acide sialique réfère à une large famille d'acides de sucres composés de neuf carbones et qui se retrouvent en position terminale de glycoconjugués exposés à la surface de plusieurs types de cellules eucaryotes. Néanmoins, la forme la plus abondante d'acide sialique est l'acide *N*-acétylneuraminique (Neu5Ac). Chez les eucaryotes, la présence de ce sucre en position terminale des glycoconjugués de surface permet la régulation de l'activation du complément à la surface de la cellule et protège donc celles-ci contre l'attaque par son propre complément[248, 249]. De plus, l'acide sialique de l'hôte joue un rôle important dans le régulation des fonctions immunitaires de plusieurs cellules.

Outre l'utilisation de ce sucre comme source de carbone et d'azote, certains pathogènes ont développé des moyens d'intégrer ce sucre à leurs composants de surface. Il est proposé que la sialylation de certains composants de surface leur confère un avantage particulier dans la résistance au système immunitaire de l'hôte, mais également dans l'interaction avec différents récepteurs cellulaires de l'hôte[248]. Parmi les pathogènes qui incorporent l'acide sialique dans leurs composants de surface on retrouve, en autres, *E. coli K1, H. influenzae,* GBS*, N. meningitidis, N. gonorrhoeae, Campylobacter jejuni* et *Candida albicans*[248].

3.2. Acquisition et biosynthèse

Les pathogènes peuvent obtenir de l'acide sialique de deux moyens différents; soit par (i) synthèse de novo, ou par (ii) l'acquisition via son environnement.

3.2.1. De novo

Chez certains pathogènes, notamment chez *S. suis* et GBS, les gènes codant les enzymes responsables de la synthèse de l'acide sialique (*neuB, neuC, neuD* et *neuA)* sont situés dans le locus codant les autres gènes de la synthèse de la CPS[49, 232, 235].

La synthèse de l'acide sialique (Neu5Ac) commence par la formation du précurseur *N*-acétylmannosamine (ManNAc) à partir du UDP-*N*-acétylglucosamine (UDP-Glc-NAc). Cette première étape est assurée par l'enzyme codée par le gène *neuC*. La deuxième étape est quant à elle assurée par l'enzyme codé par le gène *neuB*. Le ManNAc est alors condensé avec le phosphoénolpyruvate (PEP) afin de former la forme inactive de l'acide sialique, le Neu5Ac. La protéine NeuA transforme le Neu5Ac en sa forme active le CMP-Neu5Ac à l'aide du cytosine triphosphate (CTP). C'est cette forme active qui agira comme substrat à la sialyltransférase qui transférera le CMP-Neu5Ac en position terminal des chaînes polysaccharidiques formant la CPS de *S. suis* et GBS[248] (**Figure 10**).

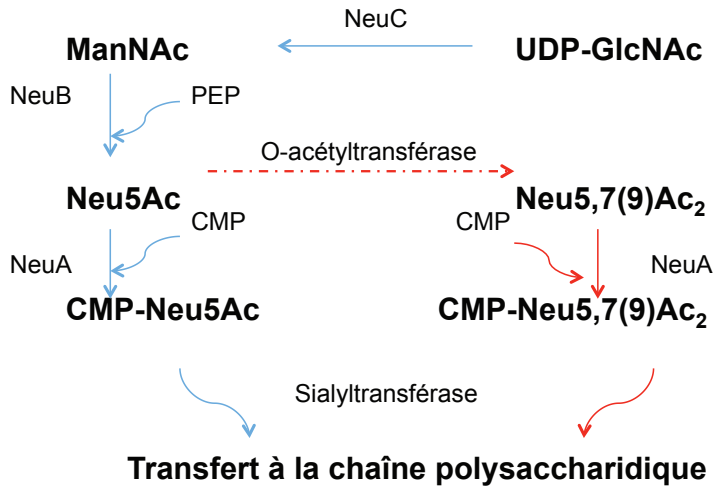

Figure 10. Modèle de la voie de synthèse de l'acide sialique et incorporation à la CPS. La biosynthèse de l'acide sialique se traduit en trois étapes; (i) Épimérization du UDP-GlcNAc en ManNAc, (ii) la condensation du ManNAc et du phosphoénolpyruvate (PEP) pour former le Neu5Ac, puis (iv) l'activation du Neu5Ac en Neu5Ac-CMP, la forme utilisée par la sialyltransférase. L'*O*-acétylation de l'acide sialique, décrit chez GBS, est schématisé en rouge. Figure modifiée à partir de Lewis & al. 2004[238].

Curieusement, la protéine codée par le gène *neuD* ne semble pas avoir de rôle dans la synthèse de l'acide sialique. Il a été démontré chez GBS que l'enzyme codé par *neuD* est responsable de l'*O*-acétylation de l'acide sialique[238]. Nous reviendrons sur l'*O*-acétylation dans la section: modification de l'acide sialique 4.5)

3.2.2. Acquisition de l'environnement

Certains pathogènes ne possèdent pas les gènes nécessaires à la synthèse de l'acide sialique. Afin de sialyler leurs composants de surface, ceux-ci sont capables de récupérer l'acide sialique libre produit par l'hôte. C'est notamment le cas de *N. gonorrhoeae*, qui, grâce à une sialyltransférase membranaire exposée à la surface, est capable de récupérer le CMP-Neu5Ac libre et le transférer directement à ses composants de surface, dont le lipopolysaccharide (LPS)[248].

Un autre moyen étonnant développé par les pathogènes pour obtenir de l'acide sialique de l'environnement est l'expression d'une trans-sialidase de surface. Cette enzyme a la particularité de pouvoir enlever l'acide sialique lié à un galactose de glycoconjugués situés à la surface des cellules de l'hôte et ensuite l'incorporer à ses propres composants. Ce moyen surprenant a été décrit chez le parasite *Trypanosoma cruzi*[248].

Finalement, un dernier moyen utilisé par certains pathogènes pour obtenir de l'acide sialique est l'acquisition du précurseur de l'acide sialique chez l'hôte. Il a été proposé que *H. influenzae* serait capable d'acquérir la forme non activée de l'acide sialique (Neu5Ac) chez l'hôte et l'internaliser afin de le modifier. L'acide sialique serait alors activé en CMP-Neu5Ac via un homologue au gène *neuA* puis incorporé dans des lipooligosaccharide (LOS)[248].

S. suis et GBS possèdent tous les deux l'ensemble des gènes nécessaires à la synthèse et au transfert de l'acide sialique et il est proposé que ces pathogènes utilisent majoritairement la voie de synthèse *de novo* afin d'obtenir de l'acide sialique. Cependant, il n'est pas impossible

que ces pathogènes seraient capables d'utiliser de l'acide sialique libre et de l'incorporer à leur CPS.

3.3. Sialylation des composants de surface

Les pathogènes *S. suis* et GBS sont les deux seules bactéries à Gram positif ayant la capacité d'incorporer de l'acide sialique dans leurs CPSs. Outre les gènes codant pour la synthèse de l'acide sialique, les loci de la CPS de *S. suis* et GBS possèdent un gène codant pour une sialyltransférase capable d'ajouter un acide sialique à un galactose ou *N*-acétylgalactosamine terminal d'une chaîne polysaccharidique. Les bactéries à Gram négatif sont quant-à-elle capable de sialyler également leur LPS/LOS.

Chez *S. suis*, aucun mutant exprimant une CPS sans acide sialique n'est disponible, ce qui complique les études sur le rôle exact de l'acide sialique dans la pathogenèse de l'infection causée par *S. suis*. À l'opposé, la perte de la sialylation de la CPS chez GBS est corrélée avec une diminution de la virulence, médiée par une réduction dans la capacité de la bactérie à résister à l'opsonophagocytose. Cependant, il a été également démontré que la sialylation de la CPS était nécessaire pour une production optimale de la CPS chez GBS. La diminution de virulence en absence d'acide sialique pourrait également être due à la diminution de l'épaisseur de la CPS à la surface de la bactérie[250].

Néanmoins, chez d'autres pathogènes à Gram négatif, certains mutants déficients dans la sialylation des composants de surface (CPS et LPS) sont généralement moins virulents. De plus, le rôle de la sialylation du LPS (en position α2,3) dans la résistance au complément et dans les propriétés antiphagocytaires a été démontré chez *N. gonorrhoaea* et *N. meningitidis*[251, 252, 253]. Il est également proposé que la faible immunogénicité des CPSs sialylées serait en partie à cause de la présence de l'acide sialique (par mimétisme moléculaire et/ou évasion immunitaire – discuté plus en détail ici-bas)[248].

3.4. Spécificité du lien de l'acide sialique

Il existe une grande variété dans la nature des liens de l'acide sialique utilisés chez les pathogènes pour la sialylation de leurs composants de surface, soit la CPS, le LPS et le LOS[248, 251]. Les pathogènes à Gram négatif *E. coli* K1 et *N. meningitidis* ont la particularité d'exprimer une CPS polysialylée[248]. Ces CPSs polysialylées sont composées d'au moins 200 résidus d'acide sialique. À l'opposé de *S. suis* et GBS, la CPS de ces pathogènes sont composées uniquement de chaînes d'acide sialique grâce à une polysialyltransférase[254]. Chez les CPSs du méningocoque de groupe B et d' *E. coli* K1, les homopolymères sont reliés par un lien α2,8, alors que chez le méningocoque du groupe C, ils sont reliés par un lien α2,9. Certaines souches d'*E. coli* (K92) sont également capables d'alterner les liens α2,8 et α2,9 pour la polysialylation de leurs CPSs. Il a été démontré que chez les sialyltransférases d'*E. coli* K1 et *E. coli* K92, la spécificité du lien exercée par chacune de ces sialyltransférases résidait dans la portion N-terminale de la protéine[255].

À l'opposé, la majorité des pathogènes pouvant sialyler leur LPS, utilise un lien α2,3. C'est notamment le cas de *N. meningitidis*, qui est d'ailleurs le seul pathogène pouvant sialyler sa CPS et son LPS. Une exception est *C. jejuni* qui utilise un lien α2,3 de l'acide sialique en position terminale et un lien α2,8 pour son LPS disialylé[248].

Chez *S. suis* et GBS, il existe une différence importante dans la sialylation de la CPS de ces deux pathogènes. La nature du lien de l'acide sialique et du galactose/*N*-acétylgalactosamine terminal est différente entre *S. suis* (α2,6)[210] et GBS (α2,3)[230] (**Figures 4 et 9**). L'effet de la nature du lien de l'acide sialique dans la virulence de ces bactéries et la modulation de la réponse immunitaire n'a jamais été étudiée. La conformation des épitopes de la CPS, notamment dépendante du type de lien de l'acide sialique, pourrait modifier différemment les interactions bactérie-hôte, et par conséquent, la pathogenèse de l'infection.

3.5. Modification de l'acide sialique : *O*-acétylation

Les acides sialiques ont la particularité de pouvoir être acétylés sur les carbones 4, 7, 8 et 9. La capacité d'acétylation de l'acide sialique des composants de surface a été décrite chez *E. coli, N. meningitidis* et GBS. Il a été démontré que l'acide sialique contenu dans la CPS de GBS peut être *O*-acétylé sur les carbones 7, 8 et 9. L'*O*-acétylation se fait d'abord sur le carbone 7, puis est transferé via le carbone 8, au carbone 9. À l'opposé, chez *E. coli* et *N. meningitidis*, le branchement des sucres en α2,8 et/ou α2,9 limite l'*O*-acétylation de l'acide sialique au niveau de certains carbones. Le gène *neuD* code également pour l'enzyme responsable de l'*O*-acétylation chez GBS. Des mutants déficients dans l'expression du gène *neuA*, nécessaire à l'activation de l'acide sialique (CMP-Neu5Ac), ont permis de démontrer une accumulation intracellulaire d'acide sialique acétylé (Neu5,7Ac_2, Neu5,8Ac_2, et Neu5,9Ac_2), suggérant que l'*O*-acétylation se produit avant l'activation et le transfert de l'acide sialique[238].

Chez *S. suis*, aucune étude n'a adressé le rôle de *neuD* dans l'*O*-acétylation de l'acide sialique capsulaire de *S. suis*. Cependant, il a été démontré que le gène *neuA* code pour un enzyme bifonctionnelle, ayant une activité de Neu5A synthétase (N-acétylneuraminate cytidylyltransférase) et d'*O*-acétylestérase. Outre l'activation de Neu5Ac en CMP-Neu5Ac, la protéine codée par *neuA* est également capable de dé-*O*-acétyler le CMP-*O*-acétylNeu5Ac. Il n'est pas connu si la sialyltransférase de *S. suis* est capable de transférer les acides sialiques *O*-acétylés, comme c'est le cas de GBS. Cependant, il a été proposé que la *O*-acétylation par NeuD et la dé-*O*-acétylation par NeuA serait un moyen pour *S. suis* de réguler la synthèse de sa CPS[256]. À titre de rappel, on sait que chez *S. suis* sérotype 2, la présence d'acide sialique est cruciale à la synthèse de la CPS.

Il est proposé que l'*O*-acétylation permet à la bactérie de générer de nouveaux épitopes capsulaires et ainsi moduler la réponse immunitaire de l'hôte, ces avantages seront discutés dans la prochaine section[238].

3.6. Interactions avec les composants de l'hôte et rôle dans la virulence

3.6.1. Résistance au complément et interactions avec les cellules

Le rôle de l'acide sialique dans la virulence de *S. suis* est encore mitigé, mais ne semble pas être un déterminant crucial à la virulence de *S. suis* sérotype 2. Il a été démontré, à l'aide de souches traitées enzymatiquement à la sialidase (afin d'enlever l'acide sialique), que les propriétés antiphagocytaires de la CPS n'étaient pas affectées lors de test *in vitro* avec des monocytes porcins. De plus, il ne semble pas avoir de corrélation entre la virulence d'une souche et la concentration en acide sialique de la CPS[257].

Un rôle indirect de l'acide sialique dans la virulence de *S. suis* a cependant été décrit dans le cas particulier d'une co-infection avec le virus de la grippe porcine H1N1. L'acide sialique présent à la surface de *S. suis* agirait alors comme un récepteur capable de lier les virus préalablement adhéré aux cellules épithéliales. La liaison entre l'acide sialique et le virus de la grippe porcine H1N1 favoriserait l'adhésion de *S. suis* aux cellules de l'hôte, mais également l'invasion de celles-ci. Dans ce cas particulier, l'acide sialique contenu dans la CPS de *S. suis* contribuerait donc à l'adhésion et à l'invasion lors de la colonisation[63].

Le rôle de l'acide sialique dans la virulence d'autres pathogènes a déjà été étudié. C'est notamment le cas de la CPS sialylée de GBS et de la CPS polysialylée d'*E. coli* K1. La CPS polysialylée d'*E. coli* K1 inhibe l'opsonisation et la phagocytose. De son côté, et tel que mentionné, la CPS sialylée de GBS protège également contre la phagocytose et augmente la résistance à l'activation du complément par la voie alternative[253]. Mais, encore une fois, dans le cas de GBS, la diminution dans l'épaisseur de la CPS en absence d'acide sialique empêche une conclusion certaine sur le rôle de ce dernier.

La sialylation du LPS contribue également à la virulence des pathogènes. Il a été démontré que chez *H. influenzae*, la sialylation du LPS contribue légèrement à l'adhésion aux

cellules épithéliales Hep-2 et joue un rôle significatif dans la résistance à l'opsonisation par le sérum humain[258].

La raison pour laquelle l'acide sialique semble être impliqué dans la virulence de GBS et non de *S. suis* n'est pas clair, néanmoins, outre la différence dans la nature du lien de l'acide sialique, la quantité d'acide sialique retrouvé dans la CPS pourrait aussi jouer un rôle. La quantité d'acide sialique chez *S. suis* est environ une fois et demi moindre que celle contenue dans la CPS des souches les moins virulentes de GBS et quatre fois moins que dans les souches virulentes de GBS[257].

3.6.2. Interactions avec les Siglecs

Les Siglecs « sialic acid binding Ig-like lectins » sont des récepteurs exprimés chez les leucocytes permettant la reconnaissance de l'acide sialique et favorisent les interactions cellulaires. Ces récepteurs permettent de reconnaître les épitopes d'acide sialique présents à la surface des cellules (majoritairement les cellules du système immunitaire) de l'hôte et de réguler la réponse immunologique envers ces cellules[259]. Il existe dix sous-groupes de Siglecs (Siglec-1 à Siglec-10). Par exemple, le Siglec-1 ou sialoadhésine est exprimé majoritairement chez les macrophages, alors que les Siglecs 2 (CD22) et 3 (CD33) se retrouvent principalement chez les cellules B et les cellules myéloïdes immatures, respectivement[260, 261]. Il est connu que les différents Siglecs ont des préférences pour la nature du lien (α2,3 ou α2,6) de l'acide sialique, mais également pour la nature des modifications de l'acide sialique, dont l'*O*-acétylation[259].

Les Siglecs permettent également aux cellules immunitaires de reconnaître les pathogènes sialylés et d'initier et/ou moduler la réponse immunitaire, dont la phagocytose. La CPS sialylée (α2,3) de GBS et les LPSs de *C. jejuni* (α2,3 et α2,8) et *N. meningitidis* (α2,3) sont capables de lier différents récepteurs Siglecs[261].

Il a été démontré que le Siglec-1 était impliqué dans la reconnaissance et l'internalisation de GBS par les macrophages, et contribuerait à restreindre la dissémination de ce pathogène[262]. De plus, une étude a rapporté que l'inhibition de l'interaction de la CPS sialylée de GBS avec le Siglec-9 mène à une diminution la réponse des neutrophiles humains, dont la capacité à former des « neutrophils extracellular traps »[263].

L'*O*-acétylation de la CPS de GBS peut également influencer la réponse des cellules immunitaires de l'hôte. Des mutants de GBS exprimant différents niveaux d'*O*-acétylation ont permis de démontrer que l'*O*-acétylation de l'acide sialique diminue l'affinité de liaison avec les siglec-5,7 et 9. Il est proposé que la diminution de l'affinité avec le Siglec-9 permettrait à GBS de diminuer le pouvoir bactéricide des neutrophiles humains *in vitro*, mais également dans le cas d'infection *in vivo*. De plus, l'*O*-acétylation empêche la désialylation de la CPS par des sialidases qui pourraient être présentes au site de l'infection, notamment dans le tractus vaginale**[263]**.

3.6.3. Recrutement du facteur H par l'acide sialique

L'acide sialique pourrait jouer un rôle également dans le recrutement du facteur H à la surface de *S. suis*. Comme mentionné précédemment, le facteur H est composé de 20 domaines. Les domaines 19-20 seraient responsables de la liaison avec l'acide sialique[264, 265]. De plus, en présence d'acide sialique, il a été démontré que l'affinité du facteur H pour le C3b était augmentée[79].

Le rôle de l'acide sialique dans le recrutement du facteur H a été caractérisé chez plusieurs pathogènes; *Pseudomonas aeruginosa*[266], *N. gonorrhoeae*[252] and *Histophilus somni*[248, 267]. Dans le cas de *N. gonorrhoeae*, il a été démontré par cytométrie en flux et par traitement à la neuraminidase que la présence de l'acide sialique augmente de quatre fois la quantité de facteur H liée à la surface de la bactérie. De plus, le facteur H recruté par l'acide sialique conserve sa fonctionnalité en inactivant le C3b présent en sa forme inactive, le iC3b[252].

Une étude récente a également confirmé la liaison du facteur H avec un glycane sialylé en α2,3. Curieusement, cette même liaison avec le facteur H n'a pas pu être confirmée avec le même glycane sialylé en α2,6. Ces résultats suggèrent que la structure spécifique médiée (notamment par le lien de l'acide sialique) induit une conformation particulière qui favorise le recrutement du facteur H[265].

Néanmoins, l'acide sialique ne semble pas être un composant recrutant le facteur H universel. Il a été démontré que chez GBS, le recrutement du facteur H était assuré par la protéine de surface Bac et que la CPS sialylée ne semblait pas pouvoir lier le facteur H[268].

La variation de la structure conformationnelle menée par l'acide sialique entre *S. suis* (α2,6) et GBS (α2,3) pourrait avoir un impact inattendu sur l'activation du complément, les mécanismes de phagocytose et la capacité bactéricide des neutrophiles.

Ainsi, basé sur cette recension de la littérature, l'**hypothèse générale** de cette thèse est que les composants de surface de *S. suis*, tels la CPS et les protéines de surface influencent directement la pathogenèse de l'infection causée par *S. suis*. Plus précisément, les hypothèses spécifiques sont que (i) La structure et la sialylation de la CPS de *S. suis* influencent ses propriétés antiphagocytaires et/ou immunomodulatrices, et (ii), les protéines de surface de *S. suis* représentent *de facto* des facteurs de virulence importants.

Les **objectifs généraux** de cette thèse sont, en premier lieu, d'investiguer l'impact de la structure et de la composition de la CPS dans les interactions hôte-pathogène et la virulence de *S. suis* et dans un second temps, étudier le rôle de certaines protéines de surface de *S. suis* ayant à présent des fonctions inconnues dans la pathogenèse de l'infection.

III. MÉTHODOLOGIE ET RÉSULTATS

ARTICLE I

Genotyping and investigating capsular polysaccharide synthesis gene loci of non-serotypeable *Streptococcus suis* isolated from diseased pigs in Canada

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Publié dans : Veterinary Research. **2017**. doi: 10.1186/s13567-017-0417-6

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

Je suis coauteur de l'article. J'ai participé activement aux expériences dans le cadre d'un stage international en Chine, à l'analyse des résultats et à la révision critique de l'article.

Abstract

Streptococcus suis (*S. suis*) is an important swine pathogen and an emerging zoonotic agent. Most clinical *S. suis* strains express capsular polysaccharides (CPS), which can be typed by antisera using the coagglutination test. In this study, 79 *S. suis* strains recovered from diseased pigs in Canada and which could not be typed using antisera were further characterized by capsular gene typing and sequencing. Four patterns of *cps* locus were observed: i. Fifteen strains were grouped into previously reported serotypes but presented several mutations in their *cps* loci, when compared to available data from reference strains; ii. Seven strains presented a complete deletion of the *cps* locus, which would result in an inability to synthesize capsule; iii. Forty-seven strains were classified in recently described novel *cps* loci (NCLs); and iv. Ten strains carried novel NCLs not previously described. Different virulence gene profiles (based on the presence of *mrp, epf*, and/or *sly*) were observed in these non-serotypeable strains. This study provides further insight in understanding the genetic characteristics of *cps* loci in non-serotypeable *S. suis* strains recovered from diseased animals. When using a combination of the previously described 35 serotypes and the complete NCL system, the number of untypeable strains recovered from diseased animals in Canada would be significantly reduced.

Introduction

Streptococcus suis is recognized as one of the most important causes of bacterial disease in post-weaned piglets worldwide, generating important economic losses to the swine industry. In addition, it is an important emerging zoonotic agent [1-3]. Clinical strains of *S. suis* generally have a capsule (capsular polysaccharide or CPS), which is the basis of the serotyping traditionally used for epidemiological studies. Thirty-five serotypes of *S. suis* (serotype 1 through 34 and serotype 1/2) were identified in the 1980s and the 1990s [4-7]. More recently, serotypes 20, 22, 26, 32, 33, and 34 have been suggested as belonging to a species different from *S. suis* [8, 9]. Strains isolated from diseased pigs primarily belong to serotype 2 in most countries, followed by serotypes 3, 4, 5, 7, 8, and 1/2 [10-12]. In some European countries, serotype 9 is also one of the most frequently recovered capsular types from diseased animals [12, 13]. Traditionally, *S. suis* is routinely serotyped by the

coagglutination test using serotype-specific antisera. However, non-serotypeable *S. suis* strains are frequently reported in many studies [12, 14-18]. Given that strains not expressing the CPS cannot be serotyped using antisera, serotyping based on molecular techniques has been proposed. Since the *S. suis* CPS is synthesized by the Wzx/Wzy pathway in the CPS locus, *wzy* genes have been demonstrated to be serotype-specific [19]. Thus, high-throughput capsular gene typing systems based on serotype-specific *wzy* genes have become attractive alternatives/complement to the existing serological tests [18, 20, 21]. However, even with the use of multiplex PCR tests, non-serotypeable strains are still commonly isolated from both clinically healthy and diseased animals [18, 22, 23].

In recent years, 17 novel *cps* loci (NCLs) were identified from non-serotypeable *S. suis* and were designated as NCL1 to 16 and serotype Chz [22-24]. Meanwhile, an 18-plex Luminex assay was also developed to detect these 17 NCLs and nearly 60% of non-serotypeable strains from healthy pigs carried one of these NCLs [22]. However, little is known about the distribution and characteristics of the *cps* loci of potentially virulent non-serotypeable strains recovered from diseased animals.

In this study, the *cps* loci of 79 Canadian non-serotypeable *S. suis* strains (as determined by the coagglutination test) recovered from diseased pigs were studied using two capsular gene typing systems [20, 22] and the genetic characteristics of the NCLs were analyzed. To elucidate the non-serotypeable mechanisms of strains grouped into previously described serotypes, the study was extended to compare their *cps* sequence to that of corresponding reference strains. Furthermore, the prevalence of minimum core genome (MCG) sequence typing group and virulence gene profile were also investigated in all 79 strains.

Results

Serotyping of strains

The 79 strains used in the present study showed auto-agglutination, poly-agglutination or non-agglutination using the reference antisera and the coagglutination test and were thus considered as non-serotypeable. All strains were then typed using our previously developed capsular gene typing systems [20, 22]. Fifteen strains (18.9%) were grouped into reference serotypes while 47 (59.4%) were grouped into 17 known NCLs. The remaining 17 strains remained non-typeable (Supplemental Table I).

Supplemental Table S1. Isolates of *S. suis* used in the present study.

Strains ID	*cps* locus type	Year of isolation	Source (disease)	MCG group	*mrp*	*sly*	*epf*	*cps* Genbank accession No.	Reads
1093407	NCL16-3	2008	lung	6	-	-	-	KX87005	SRR5177666
1097105	NCL17	2008	pleura	N	-	-	-	KX87005	SRR5177667
1114635	NCL13	2008	brain	N	-	-	-		
1119511	NCL7-1	2008	heart	6	-	-	-		
1134007	NCL3-1	2009	lung	6	-	-	-		
1145879	NCL3-1	2009	lung	6	-	-	-		
1148795	NCL3-1	2009	kidney	6	-	-	-		
1158588	NCL3-1	2009	pleura	6	-	-	-		
1177693	NCL3-1	2009	spleen	6	-	-	-		
1187537	NCL3-1	2009	spleen	6	-	-	-		
1208289	NCL19	2009	pleura	7-3	-	-	-	KX87006	SRR5177688
1212379	NCL7-1	2009	brain	6	-	-	-		
1232225	Chz-3	2010	spleen	6	-	-	-	KX87006	SRR5177689
1270831	NCL19	2010	brain	7-3	-	-	-	KX87006	SRR5177690
1289669	NCL3-1	2011	spleen	6	-	-	-		
1297150	NCL11-5	2011	brain	6	-	-	-		
1326054	NCL10	2011	brain	2	NA2	+	-		
1338413	NCL7-1	2011	brain	6	-	-	-		
1338414	NCL7-1	2011	heart	6	-	-	-		
1345589	NCL3-1	2011	pleura	6	-	-	-		
1429723	NCL13	2012	joint	7-2	-	-	-		
1469238	NCL5	2013	pleura	N	-	-	-		
1541370	NCL2-4	2013	brain	6	-	-	-		
1587992	NCL3-1	2014	brain	6	-	-	-		

1572460	NCL3-1	2014	heart	6	-	-	-		
1611502	Serotype	2014	oral	N	-	-	+	KX87007	SRR5177668
1615893	Deletion	2014	heart	4	-	-	-		
1640373	NCL1-12	2014	oral	6	-	-	-	KX87007	SRR5177711
1722945	NCL2-4	2015	lung	6	-	-	-		
78468-4	NCL18	2015	NA	3	NA1	+	-	KX87005	SRR5177692
78468-1	NCL18	2015	NA	3	NA1	+	-	KX87004	SRR5177693
78468-5	NCL18	2015	NA	3	NA1	+	-	KX87005	SRR5177694
02-B627	NCL17	2002	brain	7-1	-	+	-	KX87004	SRR5177695
1778187	NCL3-1	2015	lung	6	-	-	-		
1826706	NCL20	2016	pleura	N	-	-	-	KX87007	SRR5177663
1827702	Serotype	2016	heart	1	EU	+	+	KX87007	SRR5177664
1821899	NCL14	2015	brain	6	-	-	-		
1839679	Serotype	2016	brain	N	EU	+	+	KX87004	SRR5177665
1089122	NCL2-4	2008	spleen	6	-	-	-		
1090772	Serotype	2008	NA	4	NA1	-	-	KX87005	SRR5177669
1114193	NCL17	2008	heart	N	NA2	+	-	KX87005	SRR5177670
1109354	Serotype	2008	NA	4	-	-	-	Identical	SRR5177696
1127863	Serotype	2008	pleura	6	-	-	-	KX87005	SRR5177671
1126701	Deletion	2008	heart	N	NA1	-	-		
1126700	Deletion	2008	heart	N	-	-	-		
1139707	NCL12	2009	heart	6	-	-	-		
1144155	NCL6	2009	spleen	7-3	-	-	-		
1150386	Serotype	2009	heart	4	NA1	-	-	Identical	SRR5177679
1160406	Serotype	2009	pleura	6	-	-	-	KX87005	SRR5177674
1162453	NCL3-1	2009	brain	6	-	-	-		
1162344	NCL3-1	2009	spleen	6	-	-	-		
1167549	NCL12	2009	heart	7-2	-	-	-		
1180230	NCL17	2009	oral	7-1	-	-	-	KX87005	SRR5177675
1191976	Deletion	2009	heart	N	NA1	-	-		
1200000	NCL4	2009	umbilica	7-3	-	-	-		
1219985	NCL3-1	2010	spleen	6	-	-	-		
1218846	Serotype	2010	heart	N	-	-	-	KX87006	SRR5177677
1224887	Serotype	2010	brain	6	-	-	-	KX87006	SRR5177678
1223479	NCL4	2010	lung	7-3	-	-	-		
1239835	NCL3-1	2010	pleura	6	-	-	-		
1245801	NCL3-1	2010	brain	6	-	-	-		
1295492	NCL4	2011	lung	7-3	-	-	-		
1331267	NCL13	2011	heart	6	-	-	-		
1336897	Serotype	2011	heart	N	-	-	-	KX87006	SRR5177680
1336915	Serotype	2011	lung	N	-	+	+	KX87006	SRR5177681
1369307	NCL3-1	2012	brain	6	-	-	-		
1388972	NCL3-1	2012	spleen	6	-	-	-		
1405327	Deletion	2012	heart	N	-	-	-		

1420994	NCL12	2012	heart	7-2	-	-	-		
1424566	Serotype	2012	brain	N	-	+	+	KX87006	SRR5177684
1442767	Deletion	2012	spleen	N	-	-	-		
1449343	Serotype	2012	lung	N	-	+	-	KX87007	SRR5177685
1601397	NCL4	2014	spleen	7-3	-	-	-		
1655571	NCL4	2014	NA	7-3	-	-	-		
1655573	NCL4	2014	NA	7-3	-	-	-		
1656809	NCL4	2014	brain	7-3	-	-	-		
1725015	Deletion	2015	heart	N	-	-	-		
1761402	Serotype	2015	brain	N	EU	+	+	KX87007	SRR5177687
1790919	NCL4	2015	spleen	7-3	-	-	-		
1093407	NCL16-3	2008	lung	6	-	-	-	KX87005	SRR5177666
1097105	NCL17	2008	pleura	N	-	-	-	KX87005	SRR5177667
1114635	NCL13	2008	brain	N	-	-	-		
1119511	NCL7-1	2008	heart	6	-	-	-		
1134007	NCL3-1	2009	lung	6	-	-	-		
1145879	NCL3-1	2009	lung	6	-	-	-		
1148795	NCL3-1	2009	kidney	6	-	-	-		
1158588	NCL3-1	2009	pleura	6	-	-	-		
1177693	NCL3-1	2009	spleen	6	-	-	-		
1187537	NCL3-1	2009	spleen	6	-	-	-		
1208289	NCL19	2009	pleura	7-3	-	-	-	KX87006	SRR5177688
1212379	NCL7-1	2009	brain	6	-	-	-		
1232225	Chz-3	2010	spleen	6	-	-	-	KX87006	SRR5177689
1270831	NCL19	2010	brain	7-3	-	-	-	KX87006	SRR5177690
1289669	NCL3-1	2011	spleen	6	-	-	-		
1297150	NCL11-5	2011	brain	6	-	-	-		
1326054	NCL10	2011	brain	2	NA2	+	-		
1338413	NCL7-1	2011	brain	6	-	-	-		
1338414	NCL7-1	2011	heart	6	-	-	-		

Of the 15 strains belonging to the previously described serotypes, serotype 2 or 1/2 (n=4), which cannot be distinguished by capsular gene typing, was the most frequent, followed by serotypes 15 (*n*=3), 11 (*n*=2), and 30 (*n*=2). Serotypes 5, 17, 27 and 29 only contained a single strain (Supplemental Table I).

Of the 47 strains which were assigned to previously known NCLs, NCL3 (*n*=18) was the most prevalent, followed by NCL4 (*n*=8), NCL7 (*n*=4), NCL2 (*n*=3), NCL12 (*n*=3), and NCL13 (*n*=3). In addition, one strain each of the NCL1, 5, 6, 10, 11, 14, 16, and Chz were also found (Supplemental Table I).

Identification of four new NCLs

The remaining 17 non-serotypeable strains mentioned above were sequenced by Illumina sequencing. The *cps* locus was absent from 7 strains. The *cps* loci of the remaining 10 strains were divided into four new NCLs which were named NCL17 to 20 based on their *wzy* gene sequences. NCL17 was the most prevalent (*n*=4), followed by NCL18 (*n*=3), NCL19 (*n*=2), and NCL20 (*n*=1) (Supplemental Table I). In addition, two types of patterns were found in the four new NCLs. NCL17 and NCL18 belonged to pattern I-a, while NCL19 and NCL20 belonged to pattern I-b [19].

The sizes of these NCLs ranged from 21.34kb to 29.90kb and the percentage of G+C content varied between 33.9% and 35.1%. Fifty-nine predicted coding sequences were designated *cps* HGs. Twenty-two HGs were also present in the *cps* loci of the reference strains of known serotypes and 17 known NCLs. An initial sugar transferase gene was located in the 5' region and was classified into three HGs: HG6 (NCL20), HG8 (NCL17), and HG295 (NCL18 and NCL19). The 5' regions of four NCLs were conserved, whereas the central and 3' regions of these were highly variable (Fig. 1A).

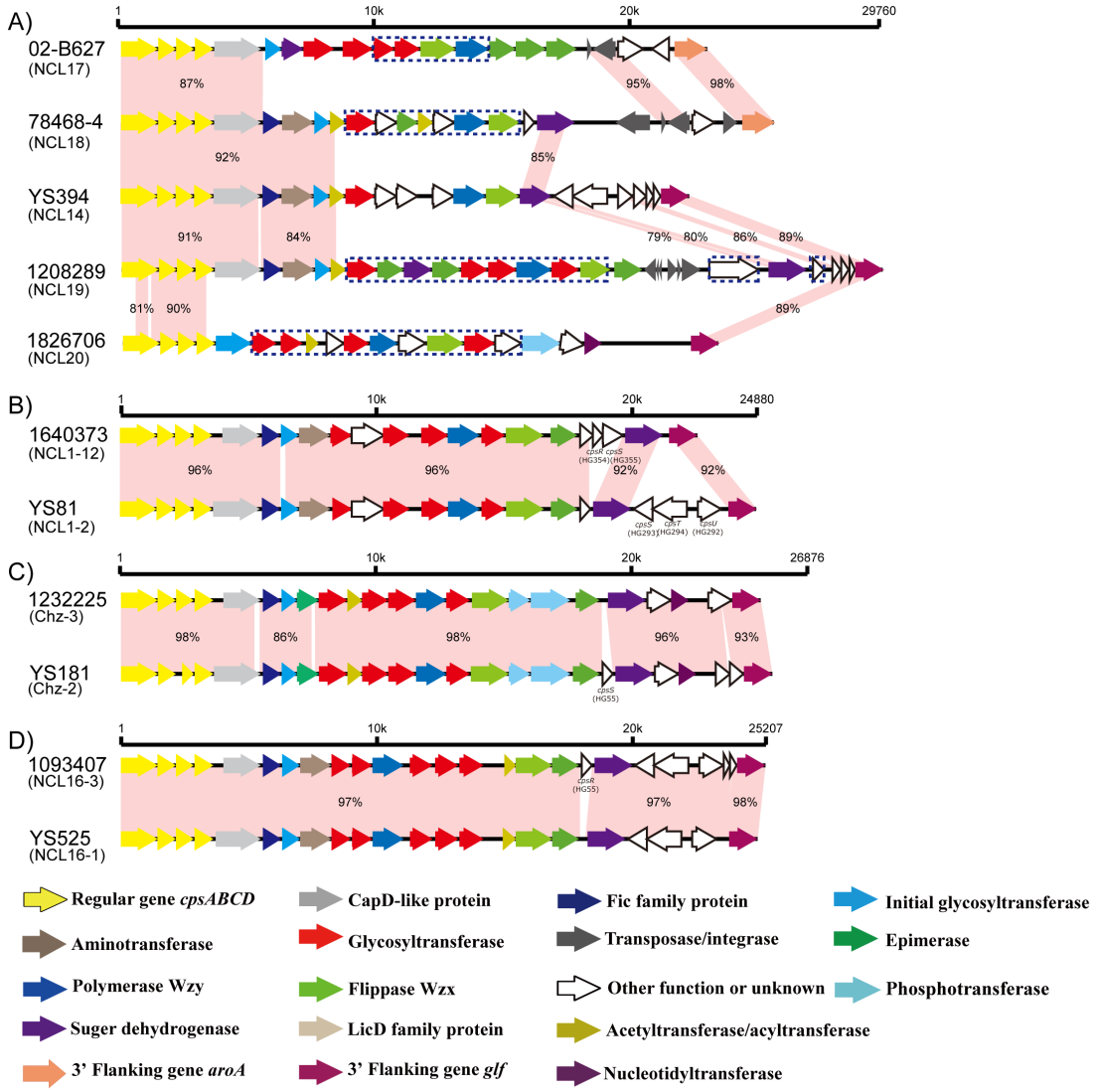

Figure 1. Comparison of the cps loci among NCL17 to 20 (A) and within NCL1 (B), Chz (C) and NCL16 (D). Each colored arrow represents the gene whose predicted function is shown in the below panel. NCL-specific genes are indicated by dotted blue lines.

Thirty-two HGs were NCL-specific. Each NCL contained 4 to 11 NCL-specific genes, with 4 HGs for NCL17, 7 HGs for NCL18, 11 HGs for NCL19, and 10 HGs for NCL20. Among these, 11 HGs encoded putative glycosyl transferases and two encoded acetyltransferases. As expected, all Wzy polymerases and Wzx flippases were NCL-specific (Supplemental Table S2).

Supplemental Table S2. Predicted product and homology group in each NCL. The font color of NCL-specific HGs is red.

Gene name	Predicted Products	HG of products	Size of the products (aa)
NCL1-12			
cpsA	Integral membrane regulatory protein Wzg	HG1	479
cpsB	Chain length determinant protein Wzd	HG2	229
cpsC	Tyrosine-protein kinase Wze	HG3	228
cpsD	Protein-tyrosine phosphatase Wzh	HG4	243
cpsE	Polysaccharide biosynthesis protein	HG5	486
cpsF	Fic family protein	HG9	238
cpsG	Glycosyl-1-phosphate-transferase	HG21	232
cpsH	Aminotransferase	HG22	405
cpsI	Glycosyltransferase	HG99	283
cpsJ	Hypothetical protein	HG296	421
cpsK	Glycosyltransferase	HG297	347
cpsL	Glycosyltransferase	HG298	355
cpsM	Wzy	HG299	415
cpsN	Glycosyl transferase	HG300	309
cpsO	Wzx	HG301	493
cpsP	UDP-glucose 4-epimerase	HG34	347
cpsQ	Hypothetical protein	HG55	173
cpsR	Hypothetical protein	HG354	130
cpsS	Hypothetical protein	HG355	262
cpsT	UDP-glucose 6-dehydrogenase	HG7	496
glf	UDP-galactopyranose mutase		369
Chz-3			
cpsA	Integral membrane regulatory protein Wzg	HG1	479
cpsB	Chain length determinant protein Wzd	HG2	238
cpsC	Tyrosine-protein kinase Wze	HG3	228
cpsD	Protein-tyrosine phosphatase Wzh	HG4	243
cpsE	Polysaccharide biosynthesis protein	HG5	486
cpsF	Fic family protein	HG9	238
cpsG	Glycosyl-1-phosphate-transferase	HG8	199
cpsH	NAD-dependent epimerase/dehydratase	HG23	286
cpsI	Putative glycosyltransferase of GT1 family	HG250	372

cpsJ	Maltose O-acetyltransferase	HG251	194
cpsK	Glycosyl transferase family 2	HG418	341
cpsL	Glycosyl transferase group 1	HG419	359
cpsM	Wzy	HG420	395
cpsN	Glycosyl transferase	HG300	308
cpsO	Wzx	HG301	480
cpsP	Phosphorylcholine transferase	HG314	283
cpsQ	Choline kinase	HG315	514
cpsR	UDP-glucose 4-epimerase	HG34	311
cpsS	UDP-glucose 6-dehydrogenase	HG7	496
cpsT	Integral membrane protein	HG312	306
cpsU	Nucleotidyl transferase family protein	HG313	229
cpsV	Hypothetical protein	HG292	306
glf	UDP-galactopyranose mutase		370

NCL16-3

cpsA	Integral membrane regulatory protein Wzg	HG1	445
cpsB	Chain length determinant protein Wzd	HG2	238
cpsC	Tyrosine-protein kinase Wze	HG3	228
cpsD	Protein-tyrosine phosphatase Wzh	HG4	243
cpsE	Polysaccharide biosynthesis protein	HG5	486
cpsF	Fic family protein	HG9	238
cpsG	Glycosyl-1-phosphate-transferase	HG21	232
cpsH	Aminotransferase	HG22	405
cpsI	Glycosyltransferase	HG211	250
cpsJ	Glycosyltransferase	HG412	264
cpsK	Wzy	HG413	405
cpsL	Glycosyltransferase	HG414	295
cpsM	Glycosyltransferase	HG415	300
cpsN	Glycosyl transferase	HG300	313
cpsO	Acetyltransferase	HG416	152
cpsP	Wzx	HG417	477
cpsQ	UDP-glucose 4-epimerase	HG34	348
cpsR	Hypothetical protein	HG55	133
cpsS	UDP-glucose dehydrogenase	HG7	496
cpsT	Hypothetical protein	HG293	247
cpsU	Hypothetical protein	HG294	451
cpsV	Hypothetical protein	HG292	314
cpsV′	Hypothetical protein	HG292	77
cpsV″	Hypothetical protein	HG292	85
glf	UDP-galactopyranose mutase		370

NCL17

cpsA	Integral membrane regulatory protein Wzg	HG1	479
cpsB	Chain length determinant protein Wzd	HG2	229
cpsC	Tyrosine-protein kinase Wze	HG3	231
cpsD	Protein-tyrosine phosphatase Wzh	HG4	243
cpsE	Polysaccharide biosynthesis protein	HG5	608
cpsF	Initial sugar transferase(Glycosyl-1-phosphate transferase)	HG8	213
cpsG	NAD-dependent epimerase/dehydratase	HG23	285
cpsH	Glycosyltransferase	HG257	400
cpsI	Glycosyltransferase	HG82	396
cpsJ	Glycosyltransferase	HG421	275
cpsK	Glycosyltransferase	HG422	334
cpsL	Wzx	HG423	459
cpsM	Wzy	HG424	430
cpsN	UDP-N-acetylglucosamine dehydratase/epimerase FnlA	HG25	346
cpsO	UDP-N-acetylglucosamine 2-epimerase FnlC	HG26	391
cpsP	Nucleoside-diphosphate-sugar epimerase FnlB	HG27	398
	Putative transposase remnant, IS66 family		61
	Integrase family protein		298
cpsQ	Hypothetical protein	HG73	329
cpsR	Polyprenyl synthetase	HG72	221
aroA	3-phosphoshikimate 1-carboxyvinyltransferase		426

NCL18

cpsA	Integral membrane regulatory protein Wzg	HG1	479
cpsB	Chain length determinant protein Wzd	HG2	229
cpsC	Tyrosine-protein kinase Wze	HG3	228
cpsD	Protein-tyrosine phosphatase Wzh	HG4	243
cpsE	Polysaccharide biosynthesis protein	HG5	608
cpsF	Fic family protein	HG9	237
cpsG	Aminotransferase	HG41	416
cpsH	Initial sugar transferase	HG295	205
cpsI	Sugar O-acetyltransferase	HG56	202
cpsJ	Glycosyltransferase	HG425	380
cpsK	Hypothetical protein	HG426	274
cpsL	Nucleoside-diphosphate sugar epimerase	HG427	279

cpsM	Acetyltransferase	HG428	202
cpsN	Hypothetical protein	HG429	271
cpsO	Wzy	HG430	420
cpsP	Wzx	HG431	402
cpsQ	Hypothetical protein	HG55	133
cpsR	UDP-glucose dehydrogenase	HG7	496
	Transposase and inactivated derivatives, IS66 family		450
	Transposase remnant, IS66 family		61
	Integrase		298
cpsS	Hypothetical protein (probable pseudogene)	HG19	288
	Transposase, IS982 family		182
aroA	3-phosphoshikimate 1-carboxyvinyltransferase		426
NCL19			
cpsA	Integral membrane regulatory protein Wzg	HG1	481
cpsB	Chain length determinant protein Wzd	HG2	229
cpsC	Tyrosine-protein kinase Wze	HG3	227
cpsD	Protein-tyrosine phosphatase Wzh	HG4	243
cpsE	Polysaccharide biosynthesis protein	HG5	608
cpsF	Fic family protein	HG9	237
cpsG	Aminotransferase	HG41	416
cpsH	Initial sugar transferase	HG295	205
cpsI	Sugar O-acyltransferase	HG56	202
cpsJ	Glycosyl transferase	HG432	401
cpsK	UDP-glucose 4-epimerase	HG433	347
cpsL	NAD dependent epimerase/dehydratase	HG434	369
cpsM	UDP-N-acetyl glucosamine 2-epimerase	HG435	374
cpsN	Glycosyltransferase	HG436	351
cpsO	Glycosyltransferase	HG437	373
cpsP	Wzy	HG438	459
cpsQ	Glycosyl transferase	HG439	359
cpsR	Wzx	HG440	393
cpsS	UDP-N-acetyl glucosamine 2-epimerase	HG121	365
	Transposase		149
	Transposase		32
	Integrase		41
	Transposase		126
	Transposase		51
	Integrase		246
cpsT	Hypothetical protein	HG441	639
cpsU	UDP-glucose 6-dehydrogenase	HG7	497

cpsV	Hypothetical protein	HG442	142
cpsW	Hypothetical protein	HG292	102
cpsW'	Hypothetical protein	HG292	95
cpsW''	Hypothetical protein	HG292	69
glf	UDP-galactopyranose mutase		370
NCL20			
cpsA	Integral membrane regulatory protein Wzg	HG1	369
cpsB	Chain length determinant protein Wzd	HG2	229
cpsC	Tyrosine-protein kinase Wze	HG3	228
cpsD	Protein-tyrosine phosphatase Wzh	HG4	242
cpsE	Initial sugar transferase	HG6	466
cpsF	Galactosyltransferase	HG443	320
cpsG	Galactosyltransferase	HG444	285
cpsH	Exopolysaccharide biosynthesis acetyltransferase	HG445	163
cpsI	Exopolysaccharide biosynthesis transcriptional activator EpsA	HG446	224
cpsJ	Cell wall biosynthesis glycosyltransferase	HG447	329
cpsK	Wzy	HG448	363
cpsL	Colanic acid biosynthesis protein	HG449	349
cpsM	Wzx	HG450	478
cpsN	Glycosyltransferase	HG451	401
cpsO	Hypothetical protein	HG452	339
cpsP	Choline kinase	HG315	509
cpsQ	Integral membrane protein	HG312	306
cpsR	Nucleotidyl transferase family protein	HG313	227
glf	UDP-galactopyranose mutase		370

Determining the subtypes of NCLs

NCL2, NCL3, NCL7, and NCL11 strains were found to belong to a single subtype; NCL2-4, NCL3-1, NCL7-1, and NCL11-5, respectively. Genetic heterogeneity was not found within strains of NCL12, NCL15, and NCL17 to 20 (Supplemental Table II).

(i) NCL1. Strain 1640373 could not be classified into any known NCL1 subtype and was sequenced by Illumina sequencing, named as NCL1-12. The replacement of HG293, HG294, and HG292 by the HG354 and HG355 was found in its three side regions (Fig. 1B).

(ii) Chz. Compared to the reference strain Chz-2, the deletion of HG55 was found in strain 1232225, named as Chz-3 (Fig. 1C).

(iii) NCL16. Compared to the reference strain YS525 (NCL16-1), the insertion of HG55 was found in strain 1093407, named NCL16-3 (Fig. 1D).

Mutations in the cps loci of strains belonging to previously described serotypes.

The 15 strains that were negative by coagglutination test but positive by multiplex Luminex assay for the reference serotypes were further analyzed. Comparing to the *cps* locus of the corresponding serotype reference strains, insertions and deletions were found in the serotype 5, 11, 15, 17, and 30 strains. The *cps* loci of four serotype 2 or 1/2 strains and one serotype 27 strain were intact and small-scale mutations were detected in these (Table II).

(i) Serotype 2 or 1/2. Compared to the serotype 2 reference strain P1/7 (GenBank accession number BR001000), all four strains had a 33 bp insertion in *wxy* genes and four strains had single-nucleotide substitutions in *wzx* genes. The single-nucleotide substitutions in glycosyltransferase genes and a 27 bp deletion in the side-chain formation gene were also found in five strains (Table II).

(ii) Serotype 5. Compared to the serotype 5 reference strain 11538 (GenBank accession number BR001003), the deletions of HG17 to HG19 at the 3' end were found in strain 1218846 (Fig.2A).

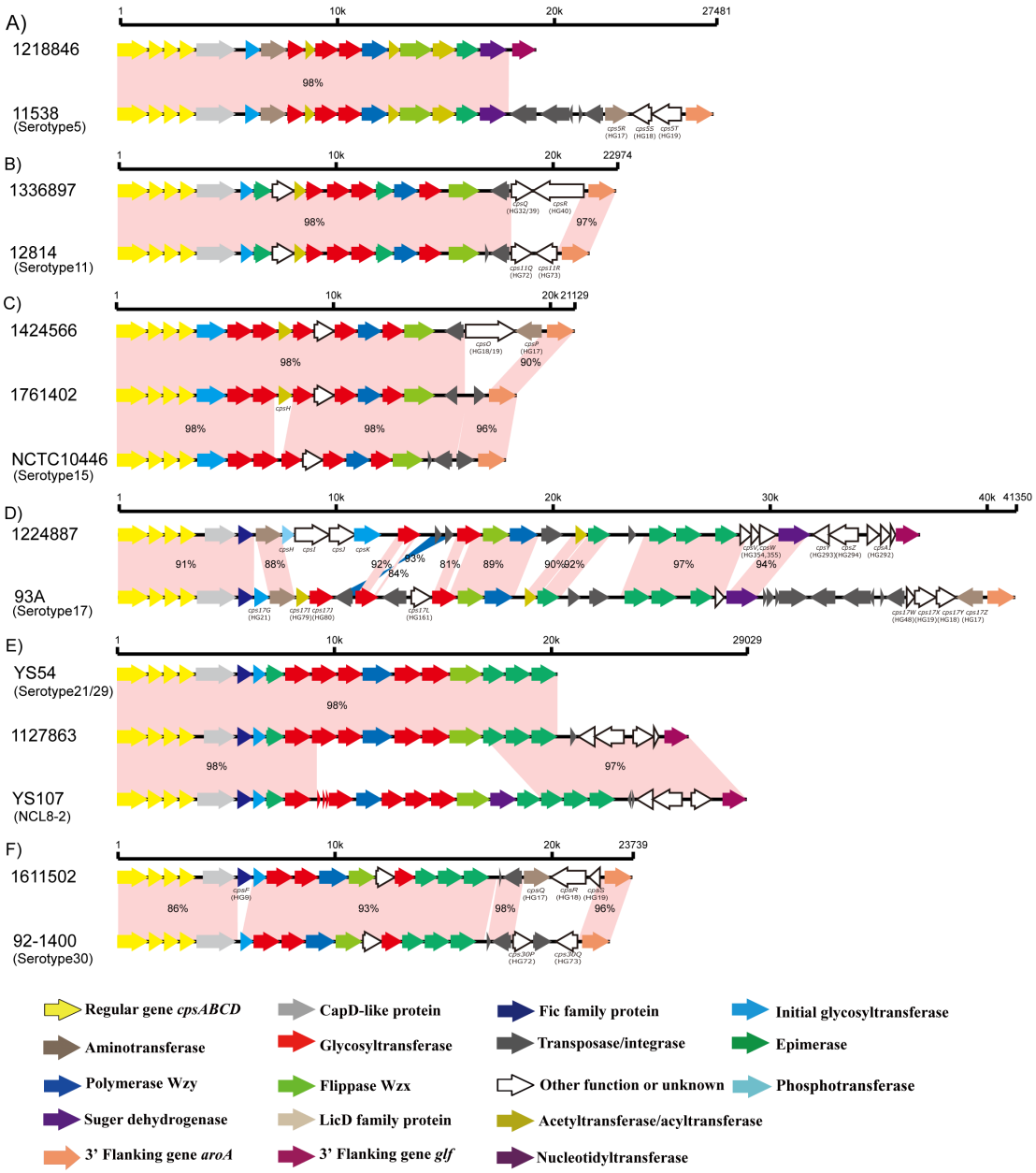

Figure 2. Comparison of the cps loci within serotype 5 (A), serotype 11 (B), serotype 15 (C), serotype 17 (D), serotype 21/29 (E) and Chz (F). Each colored arrow represents the gene whose predicted function is shown in the below panel.

(iii) Serotype 11. Compared to the serotype 11 reference strain 12814 (GenBank accession number AB737819), HG72 and HG73 were replaced by HG32 and HG40 in strains 1336897 and 1336915. In addition, the nucleotide substitutions (TA→CC) of the termination codon of HG32 were found in the *cpsQ* gene of strains 1336897 and 1336915, which resulted in the chimeric HG32/HG39 gene (Fig. 2B).

(iv) Serotype 15. Two types of variations were found within this serotype. Strains 1424566 and 1449343 possessed identical *cps* sequences. A novel HG (*cps*H, putative acetyltransferase) was inserted between HG33 and HG77, and the insertions of HG19 and HG17 at the 3' end were found in two strains (Table I). Moreover, the transversion (T→G) was found at the site of the termination codon of HG19, which resulted in the chimeric HG18/HG19 gene in two strains. Compared to strains 1424566 and 1449343, HG18 and HG17 were replaced by a transposase in strain 1761402 (Fig. 2C).

Table I. Information of the novel HGs inserted *cps* loci of strains belonging to reference serotypes

Strain ID	***cps* locus type**	**Gene name**	**Predicted products**	Similar protein, species (GenBank accession number)	**Coverage/ identity (%)**
1424566, 1449343, 1761402	Serotype 15	*cpsH*	Acetyltransferase	Maltose O-acetyltransferase, *Lactobacillus reuteri* (CUR43586.1)	96/68
1224887	Serotype 17	*cpsH*	UDP-phosphate galactose phosphotransferase	UDP-phosphate galactose phosphotransferase, *Sphaerochaeta pleomorpha* (WP_014270310.1)	99/57
		cpsI	Hypothetical protein	Biotin carboxylase, *Sphaerochaeta pleomorpha* (WP_014270309.1)	68/48
		cpsJ	Biotin carboxylase	Biotin carboxylase, *Ruminococcus sp.* (CBL19829.1)	99/60
		cpsK	Glycosyltransferase	Glycosyltransferase family 1 protein, *Gallibacterium anatis* (WP_065231950.1)	92/58

(v) Serotype 17. Compared to the serotype 17 reference strain 93A (GenBank accession number AB737824), two deletions (HG21 and HG161) and two insertions (HG354 and HG355) were found. Furthermore, HG79 and HG80 were replaced by a putative phosphotransferase, a putative hypothetical protein, a putative biotin carboxylase, and a putative glycosyltransferase (initial sugar transferase), which were not assigned to any previous homology group (Table I). Moreover, the replacement of HG48, HG17, HG18, and HG19 by HG293, HG294, and HG292 was also found (Fig. 2D).

(vi) Serotype 27. Compared to the serotype 27 reference strain 89-5259 (GenBank accession number AB737831), the single-nucleotide substitutions and small-scale indels in glycosyltransferase genes, *wzx* gene, and side-chain formation gene were found (Table II).

(vii) Serotype 29. Compared to strain YS54 agglutinated with both serotypes 21 and 29 antisera (GenBank accession number KC537387), the insertions of a transposase gene, HG293, HG294, and HG292 on the 3'-side were found in strain 1127863 (Fig. 2E).

(viii) Serotype 30. Compared to the serotype 30 reference strain 92-1400 (GenBank accession number AB737834), insertion of HG9 was found in strains 1611502 and 1839679. Moreover, HG72, transposase gene, and HG73 were replaced by HG17, HG18, and HG19 (Fig. 2F).

Table II. Mutations in glycosyltransferase, side-chain formation, *wzy* and *wzx* genes of serotype 2 or 1/2 representative strains and a serotype 27 strain.

Strain	Affected gene(s)	Types of mutations	Affected nucleotide(s) [Affected amino acid]
1827702	*cps2E*	Missense	A61G [Thr21Ala]
	cps2I	Insertion	IS element: 33bp
	cps2N	Deletion	27bp
1090772	*cps2E*	Missense	A61G [Thr21Ala]
	cps2F	Missense	A149G [Asp50Gly]
		Missense	A1047T [Leu349Phe]
	cps2I	Insertion	IS element: 33bp
	cps2N	Deletion	27bp
	cps2O	Missense	C859A [Arg287Ser]
1160406	*cps27E*	Missense	A506G [Asp169Gly]
		Missense	T508A [Ser170Thr]
		Missense	A513T [Glu171Asp]

	Missense	A522T [Lys174Asn]
	Missense	A524T A525T [Lys175Ile]
	Missense	A541C C543G [Ile181Leu]
	Missense	A553G [Ile185Val]
	Missense	G617T T618G [Ser206Met]
	Missense	A623T [Tyr208Phe]
	Missense	T633G [Leu211Val]
	Missense	C640T A642C [Leu214Phe]
	Missense	A651T [Glu217Asp]
	Missense	C692T [Ser231Leu]
	Missense	G706A A708G [Ala236Thr]
	Missense	G874A A876T [Val292Ile]
	Missense	T905C [Val302Ala]
	Missense	A922C A924G [Lys308Gln]
	Missense	A941C G942A [Lys314Thr]
	Missense	A967G [Ile323Val]
	Missense	A997C G999C [Met333Leu]
	Missense	G1000A C1001G T1002C [Ala334Ser]
	Missense	A1174C A1175G [Lys392Arg]
	Missense	G1186A G1187T T1188G [Gly396Met]
	Missense	A1272C [Glu424Asp]
	Missense	A1279C A1280G [Lys427Arg]
	Missense	G1288T T1290A [Val430Leu]
	Missense	A1292C [Glu431Ala]
	Missense	G1324A A1326T [Val442Ile]
	Missense	A1357T T1359G [Ile453Leu]
	Missense	A1360T A1361T [Lys454Leu]
cps27F	Deletion	27bp
	Missense	A662T [His221Leu]
cps27G	Insertion	IS element: 21bp
cps27I	Missense	G483C [Trp161Cys]
	Missense	C513A [Asp171Glu]
	Missense	A611G [Gln204Arg]
cps27L	Deletion	57bp
cps27M	Missense	C1373T C1374T [Ala458Val]
	Missense	C1375T [Leu459Phe]

Variations of chromosomal loci

In a previous study, the chromosomal loci of *cps* gene clusters of reference serotype 5 and 17 strains were classified into pattern I-a [19]. In the present study, strains 1218846 (serotype 5) and 1224887 (serotype 17) were classified into pattern I-b (Fig. 2A and 2D, respectively).

MCG typing

The majority of the 79 strains were clustered in the MCG group 6 (44.3%, 35/79 strains), followed by ungroupable (24%, 19/79 strains), and group 7 (20.2%, 16/79 strains). MCG groups 4, 3, 2, and 1 also contained four, three, one, and one strains, respectively (Supplemental Table I).

Identifying genotypes of *mrp, epf,* and *sly*

Twelve strains were *mrp* positive. Frameshift mutations at 2740 bp from the reported initiator ATG codon were present in the *mrp* gene of strain 1114193, which resulted in premature stop codons. Eleven other strains contained intact full-length *mrp* gene copies and may express MRP. Based on the *mrp* subtypes reported in North America (NA) [11], the sequences of *mrp* were grouped into one of three subtypes, EU (European, n=3), NA1 (n=7), or NA2 (n=1). Only twelve strains contained the *sly* gene and 6 strains were positive for *epf*. There were eight genotypes of *mrp*, *epf*, and *sly*, primarily based on *mrp* variation**:** most of the strains in this study (n=62) were $mrp^{-}sly^{-}epf^{-}$, followed by $mrp^{NA1}sly^{-}epf^{-}$ (n=4), $mrp^{NA1}sly^{+}epf^{-}$ (n=4), and $mrp^{EU}sly^{+}epf^{+}$ (n=3). It is noteworthy that the latter strains were serotype 2 or 1/2, serotype 15 and serotype 30, as revealed by the 32-plex Luminex assay**.** In addition, $mrp^{-}sly^{+}epf^{+}$ (n=2), $mrp^{-}sly^{+}epf^{-}$ (n=2), $mrp^{NA2}sly^{+}epf^{-}$ (n=1), and $mrp^{-}sly^{-}epf^{+}$ (n=1) genotypes were also found (Supplemental Table I).

Discussion

In addition to the traditional 35 serotypes originally described for *S. suis*, 17 NCLs have recently been reported in non-serotypeable *S. suis* strains isolated from healthy animals using high-throughput typing systems and online bioinformatics [22, 23]. However, the genetic characteristics of *cps* loci in potentially virulent non-serotypeable *S. suis* strains recovered from diseased animals are still scarce.

In the present study, the *cps* loci of 79 Canadian non-serotypeable strains isolated from the internal organs of diseased pigs were analyzed. Non-serotypeable strains are frequently isolated from diseased animals in this country [34]. Based on previous gene typing and sequencing results [22, 35], the non-serotypeable phenotype may be attributed to one of three causes: **(i)** strains belonging to previously described serotypes harboring mutated *cps* loci causing loss of capsule expression or antigenic variation; **(iii)** strains without *cps* locus completely losing their ability to synthesize capsule; or **(ii**i**)** strains with not-previously described NCL referring to novel serotypes.

In this study, 15 non-serotypeable strains could be grouped into reference serotypes by the 32-plex Luminex assay. To elucidate the lack of positive identification by the coagglutination test, we further sequenced and compared their *cps* loci to those of corresponding reference strains. Previous studies showed that replacements and large indels, as well as small-scale mutations of *cps* genes, caused phenotypical changes in agglutination tests [21, 36-38]. We also found similar mutations in the *cps* loci of strains tested.

HG17, HG18, HG19, HG32, HG39, and HG40, which were present in the *cps* loci of the reference strains belonging to serotypes 1, 2, 4, 5, 7, 14, 17, 18, 19, 23, and 1/2, were detected in the *cps* loci of strains in the present study belonging to serotypes 11, 15, and 30. Moreover, chimeric HG18/HG19 and HG32/HG39 genes were found in serotype 11 and 15 strains. It is noteworthy that HG292, HG293, HG294, HG354, and HG355, only present in the *cps* loci of NCLs, were also detected in the *cps* locus of strain 1224889, typed herein as serotype 17. In addition, some genes which were never before assigned to any HG were found to be inserted

in the *cps* loci of strains belonging to serotypes 17 and 15. The sequence differences between strains NCL8-2 and 1127863 were mainly caused by the replacement of 8 NCL-specific HGs in the center of NCL8-2 and by 6 HGs in 1127863. The replacement and insertion activities may indicate recombination events or horizontal gene transfer between the *cps* loci of *S. suis* strains, probably leading to antigenic variations that would be beneficial to *S. suis* in the course of infection or through immunity evasion.

Comparing to the *cps* loci of their corresponding reference strains, only small-scale mutations were observed in four strains typed as serotype 2 or 1/2 by the 32-plex Luminex. Previous study revealed that all serotype 2 and all serotype 14 strains had a G nucleotide at position 483 of the *cpsK* gene, while all serotype 1 and all serotype 1/2 strains (including 13 serotype 1/2 strains recovered in Canada) contained either a C or T at that nucleotide position [39]. In present study, all four strains had a G nucleotide at position 483 of the *cpsK* gene. We postulated that they were most probably non-encapsulated serotype 2 strains. A previous study reported that single-nucleotide substitutions and frameshift mutations in two glycosyltransferase genes (*cps2E* and *cps2F*) were the main causes of capsule loss in serotype 2 strains. Moreover, mutations in the genes involved in side-chain formation (*cps2J* and *cps2N*), *wzy* (*cps2I*), and *wzx* (*cps2O*) also appeared to be lethal to serotype 2 strains [36]. It may be hypothesized that the missense mutations and small scale indels found in these genes in strains of the present study also had a deleterious effect on the capsular expression. Indeed, high hydrophobic indexes have been obtained with these strains (unpublished data), which strongly suggest lack of capsule expression [15, 40]. Although non-encapsulated *S. suis* strains had originally been considered to be avirulent, they are frequently isolated from cases of endocarditis; as such, non-encapsulation could be, under certain circumstances, beneficial for *S. suis* in the course of such infections [36, 41]. In some cases, non-encapsulated strains resulting from small point mutations may switch to a capsulated phenotype in vivo [42]. Interestingly, small-scale mutations or clear deletions of *cps* loci were also found in an additional eight strains, which are also probably non-encapsulated. Finally, strains without *cps* locus completely losing their ability to synthesize capsule were also found in this study. It is possible that these strains are not able to reverse the encapsulated phenotype. The biological and pathological significance of these non-encapsulated strains need to be further evaluated.

Although never described, it is not impossible that some strains lose the capsular phenotype after in vitro culture.

In this study, 60% of non-serotypeable strains carried one of the recently described NCLs. The most common NCLs were 3, 4, 7, and 17, whereas in a previous study with strains recovered from healthy pigs in China, the most common NCLs were 1, 2, 3, and 7 [22]. Differences may be due to the geographical origin of strains (Canada vs. China) and/or their virulence potential (strains from diseased or clinically healthy animals). Since many strains of NCL3 have been identified in this study, further research on its virulence potential should be performed. In addition, and similarly to a previous study [22], high diversity within the same NCL was observed. The *S. suis* species is composed of phenotypically and genetically diverse strains. Host specificity and ecological environment may contribute to this diversity. The *cps* loci could provide important information regarding the ecology of strains. The differences in dominant NCLs between clinical strains from Canada and field strains from China and the emergence of novel NCLs or subtypes in clinical strains from Canada are expected.

In this study, new NCLs (CNL17 to 20), distributed in 10 strains, are reported for the first time. These NCLs possess completely different Wzy and transferases from those of the previously reported serotypes and NCLs, which in turn may express unique oligosaccharide structures and antigen identities. It is noteworthy that, taking into consideration all NCLs, more than 70% of non-typeable strains could now be typed. The use of the complete serotyping and NCL typing system would considerably reduce the number of non-typeable strains recovered from diseased animals in Canada.

The presence of some genes, such as *mrp*, *epf*, and *sly*, has been associated with virulence [43, 44]. Three distinct *mrp* genotypes have been reported so far and NA1 was the dominant genotype in *S. suis* strains recovered from diseased pigs in the USA [11]. In the present study, 11 strains possessed an intact *mrp* gene and NA1 genotype whereas three strains harbored the EU genotype. One of latter strains was typed as being a serotype 2 or 1/2 by the 32-plex Luminex but, as mentioned above, it is probably a real serotype 2 as shown by the presence of a G nucleotide at position 483 of the *cpsK* gene. The fact that most mrp^+, epf^+, and sly^+

Eurasian serotype 2 strains belong to the clonal complex 1 [12] also indicated the strain is most probably a non-encapsulated serotype 2 strain with an Eurasian profile [11] that might have been introduced to North America through the importation of animals. In fact, it has been reported that up to 5% of serotype 2 strains recovered in the United States are ST1 and probably originated from Europe [11]. Although the most prevalent virulence gene profile was *mrp*$^{-}$*sly*$^{-}$*epf*$^{-}$, 17 strains contained at least one of these three genes. The relevance of these virulence markers in strains of serotypes different from serotype 2 is still controversial.

The most prevalent MCG groups amongst the strains harboring NCLs were the groups 6 and 7, which had been described as being the most ancient groups in the *S. suis* population [30]. This indicates that their *cps* loci have existed for a long time and play important roles in the serotype diversity of *S. suis* population. The most prevalent MCG groups amongst the strains harboring mutated *cps* loci of previously described serotypes and the strains losing their *cps* loci were MCG ungroupeable. These strains possibly had a more significant recombination history that prevented them from being reliably assigned; meanwhile these recombination events may facility the mutations and loss of their *cps* loci.

Conclusion

In conclusion, this study provides further insight in understanding the *cps* diversity of *S. suis* and may contribute to future epidemiological studies that will allow characterization of potentially virulent and previously non-serotypeable strains isolated from diseased animals. Use of the 35 serotype-based system complemented with the NCL typing system would significantly reduce the number of untypeable strains recovered from diseased animals in Canada. Further studies with *S. suis* strains isolated in other countries are needed.

Materials and methods

Bacterial strains and chromosomal DNA preparation.

A total of 79 *S. suis* strains isolated from diseased pigs on non-related farms in Canada were used in this study **(Supplemental Table S1)**. All strains have been isolated from primary affected organs of clinically diseased pigs, including brain (meningitis; n=18), heart (endocarditis; n=18), multiple organs (septicemia; n=14), pleura (polyserositis; n=9), lungs (pneumonia; n=9) and joints (arthritis; n=1). For a very few isolates, the information was not available, but they were all recovered from diseased animals with a primary diagnosis of *S. suis* infection. All isolates were serotyped using the coagglutination test [25]. Chromosomal DNA was prepared from all strains as previously described [21]. The species identity of the 79 strains was determined to be *S. suis* by amplification of the 16S rRNA, *recN*, *gdh*, and *thrA* genes [20, 26-28].

Capsular gene typing.

The *cps* locus type of the 79 strains was identified by the 32-plex and 18-plex Luminex assays previously reported [20, 22]. The subtypes of known NCLs were determined based on the arrangement of subtype-specific homology groups (HGs) and transposases [22, 23].

Sequencing cps loci and bioinformatics analyses.

Seventeen strains which could not be grouped using the 32-plex and 18-plex Luminex assays and 3 strains which could not be grouped into known subtypes, as well as 15 strains which were grouped into reference serotypes, were sequenced by Illumina sequencing as previously described [23]. Each *cps* locus sequence was extracted from the draft genome sequence and was analyzed using the same bioinformatics methods described in previous studies [19, 22, 23]. The products of the *cps* genes were assigned to novel HGs if both of the global match regions and identity of the amino acid or nucleotide sequences were below 50% when compared to the 420 currently known HGs of the 35 reference serotypes and 17 NCLs. The novel HGs were assigned numerical values from HG421 onwards [19, 22, 23]. Novel HGs that were present in all strains of a given NCL were identified as NCL-specific HGs. The

strains harboring the same *wzy* were clustered into the same NCL. The Artemis comparison tool (ACT) was used to visualize the data [29].

MCG typing and PCR assays for *mrp* (muramidase released protein), *sly* (suilysin) and *epf* (extracellular protein).

MCG sequence typing was performed using PCR amplification and DNA sequencing as previously described [30, 31]. The full-length *mrp* gene was amplified and sequenced using a previously described method [11]. Amplification of the *sly* and *epf* genes was performed according to methods previously described [32, 33].

Nucleotide sequence accession number.

Sequences of *cps* loci obtained in this study were deposited in GenBank under the accession numbers KX870047-KX870056, KX870058-KX870064, KX870067-KX870072, and KX870074-KX870076. Reads of the sequenced strains were deposited in GenBank under accession number SRR5177663-SRR5177696 and SRR5177711. All accession numbers can also be found in Supplemental Table I.

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ARTICLE II

Role of the capsular polysaccharide as a virulence factor for *Streptococcus suis* serotype 14

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Publié dans: Canadian Journal of Veterinary Research. **2015**. 79 (2), 141–146

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 et à la réalisation des expériences, à l'analyse des résultats et à l'écriture de l'article.

Abstract

Streptococcus suis is an important swine pathogen and a zoonotic agent causing meningitis and septicemia. Although serotype 2 is the most virulent type, serotype 14 is emerging, and understanding of its pathogenesis is limited. To study the role of the capsular polysaccharide (CPS) of serotype 14 as a virulence factor, we constructed knockout mutants devoid of either *cps14B,* a highly conserved regulatory gene, or *neu14C,* a gene coding for uridine diphospho-N-acetylglucosamine 2-epimerase, which is involved in sialic acid synthesis. The mutants showed total loss of the CPS with coagglutination assays and electron microscopy. Phagocytosis assays showed high susceptibility of mutant Δ*cps14B*. An *in vivo* murine model was used to demonstrate attenuated virulence of this non-encapsulated mutant. Despite the difference in the CPS composition of different serotypes, this study has demonstrated for the first time that the CPS of a serotype other than 2 is also an important antiphagocytic factor and a critical virulence factor.

Introduction

Streptococcus suis, a Gram-positive bacterium, is an important swine pathogen causing meningitis, septicemia, endocarditis, arthritis, and other infections (1). It is responsible for economic losses to the swine industry and is considered an emerging zoonotic agent causing mainly meningitis and septic shock in humans (2). Thirty-five serotypes based on capsular epitopes have been described (1). Serotype 2 is considered the most virulent and has several reported virulence-associated factors implicated in infection (3). Among these factors, the capsular polysaccharide (CPS) is one of the most important, playing a crucial role in the infection (4). In fact, almost all studies in the literature on virulence factors have been done with serotype 2 strains (3).

In addition to serotype 2, serotype 14 is highly virulent and represents serious health and economic problems in several countries, such as Thailand (in humans) and those of the United Kingdom (in swine) (5,6). Swine and human cases due to this serotype have also been described in Canada (7,8). Recent chemical analyses of the CPS of serotypes 2 and 14 revealed differences in sugar composition. For example, no rhamnose residue was found in the

serotype 14 CPS (9). A side chain containing sialic acid (coded by the genes *neuA* to *neuD*) and an α-2,6-sialyltransferase are present in the CPS of both serotypes (10). Bacterial sialic acid has been implicated as a virulence factor for several pathogens, such as the closely related group B *Streptococcus* (GBS) (11). However, mutants lacking genes involved in sialic acid synthesis were found to have poorly encapsulated or non-encapsulated phenotypes for GBS and *S. suis* serotype 2, respectively, which complicates the study of the sialic acid moiety as a virulence factor (12).

Since no studies had yet been carried out on the role of CPS as a virulence factor for any *S. suis* serotype other than serotype 2, we constructed a serotype 14 isogenic knockout mutant devoid of a highly conserved regulatory gene, *cps14B*. In addition, we studied the effect of the absence of sialic acid on the expression of the whole CPS of serotype 14 by constructing a mutant deficient in the *neu14C* gene, which codes for uridine diphospho-N-acetylglucosamine 2-epimerase, an enzyme involved in sialic acid synthesis.

Results and discussion

The coagglutination serotyping test is an easy and rapid method to indirectly observe the presence of the capsule in *S. suis* isolates. In this study, both Δ*cps14B* and Δ*neu14C* mutants showed a clear negative result: no agglutination reaction with any typing antiserum. The wild-type strain, DAN13730, showed a strong and fast reaction with serotype 14-specific antiserum. As depicted in Figure 1, TEM showed a thick capsule surrounding the wild-type strain (A) and a complete absence of CPS structure for both mutants (B, C). The gene deletions were complemented in Δ*cps14B/cps14B* and Δ*neu14C/neu14C* to partially restore CPS production, as expected (D, E). Smith et al (20) demonstrated the importance of the glycosyltransferase gene *cps2EF* and a gene involved in chain-length determination *(cps2B)* in the production of CPS for serotype 2, using mutants that resulted in a capsule-deficient phenotype. The non-encapsulated Δ*cps14B* mutant showed that *cps2B* is also required for CPS synthesis in serotype 14. Lack of the sialic acid synthesis gene prevented CPS production in the Δ*neu14C* mutant. This result suggests that, despite differences in CPS

composition, sialylation of the *S. suis* serotype 14 CPS repeating units is crucial for CPS exportation or polymerization, as has been reported for serotype 2 (12). Unfortunately, it is still not possible to precisely study the role of sialic acid in the virulence of both serotypes.

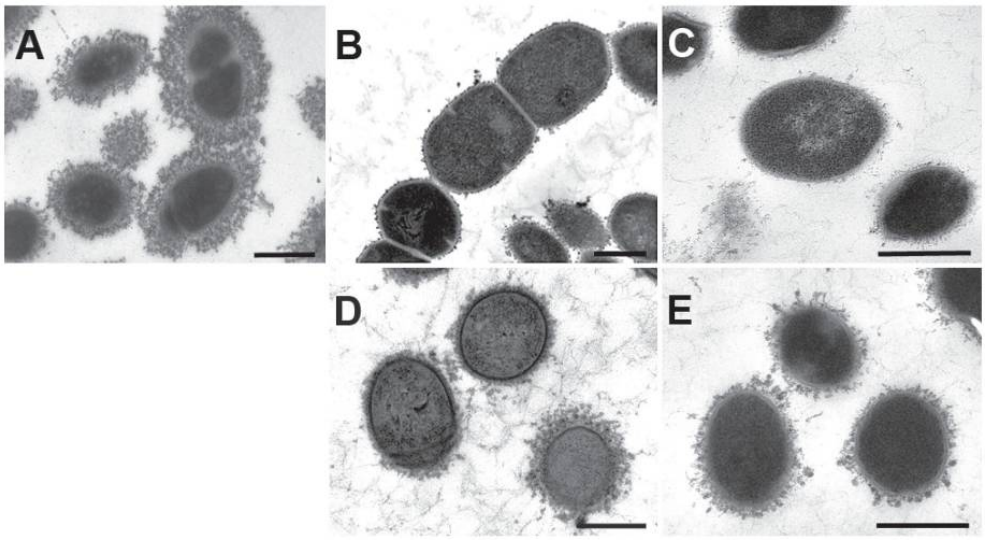

Figure 1. Transmission electron micrographs showing the expression of capsular polysaccharide (CPS) by the wild-type strain of *Streptococcus suis* serotype 14 and its derived mutants. The CPS was labelled with polycationic ferritin. The wild-type strain, DAN13730 (A), is surrounded by a thick capsule, whereas the Δ*cps14B* and Δ*neu14C* mutant strains (B and C, respectively) are non-encapsulated. The complemented strains Δ*cps14B/cps14B* (D) and Δ*neu14C/neu14C* (E) show an intermediate state of CPS production. Bars = 0.5 μm.

Since both mutants were non-encapsulated, further experiments were carried out with Δ*cps14B* alone to investigate the role of CPS in the pathogenesis of *S. suis* serotype 14 infections. Bacterial clearance by phagocytic cells represents an important host mechanism for defending against bacterial infection. To evaluate the susceptibility to phagocytosis of the capsule-deficient mutant Δ*cps14B,* bacteria were incubated with the J774A.1 murine macrophage-like cell line. As expected, the control serotype 2 strain P1/7 and its capsule-deficient mutant Δ*cps2F* were poorly and highly internalized by macrophages, respectively (Figure 2). The wild-type serotype 14 strain DAN13730 was highly resistant to phagocytosis, and only a few bacteria were internalized. In contrast, the serotype 14 non-encapsulated mutant Δ*cps14B* was significantly more internalized than its wild-type strain ($P < 0.05$). The Δ*cps14B/cps14B* complemented strain, in which the CPS was partially restored, was less

internalized than the Δ*cps14B* mutant strain but still more phagocytosed than the wild-type strain ($P < 0.05$). These results are similar to those previously reported for serotype 2 strains with use of the RAW264.6 murine macrophage cell line and porcine alveolar macrophages (12,20,21). Overall, the high phagocytosis susceptibility of Δ*cps14B* compared with the wild-type strain shows the essential role of the CPS in phagocytic resistance to *S. suis* serotype 14. Interestingly, the wild-type serotype 14 strain demonstrated even greater resistance to phagocytosis than the serotype 2 strain in this *in vitro* model ($P < 0.05$).

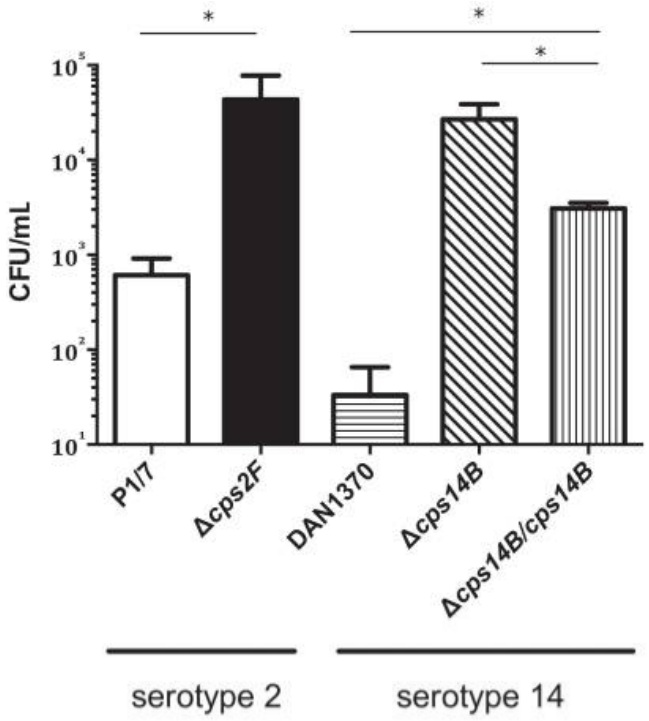

Figure 2. Phagocytosis of *S. suis* serotypes 2 and 14 by murine macrophages. Results for the well-encapsulated virulent serotype 2 strain P1/7 and its previously obtained isogenic capsule-deficient mutant Δ*cps2F* (14) are depicted for comparison only. Bacteria, 1×10^7 colony-forming units (CFU)/mL, were incubated for 60 min with J774A.1 murine macrophage-like cells, at a multiplicity of infection of 100, and gentamicin/penicillin G was used to kill any extracellular bacteria remaining after incubation. Intracellular counts were done after 3 washes and cell lysis with water. Results represent the mean count + the standard error in 4 independent experiments. Each asterisk indicates a significant difference between strains ($P < 0.05$) according to the Mann–Whitney rank-sum test.

The role of the CPS in the virulence of a serotype other than 2 was also demonstrated *in vivo*. In the first mouse model for a serotype 14 strain, we investigated the

effect of capsule loss using an *in vivo* CD1 murine model of *S. suis* infection. All 15 mice inoculated with the wild-type strain presented severe clinical signs of infection and died within the first 36 h (Figure 3A). The animals died relatively fast and mainly from septicemia and septic shock. Reducing the dose by 1 log resulted in complete absence of clinical signs and death. In contrast to the results with serotype 2, no cases of meningitis were observed, suggesting that the 2 serotypes do not behave identically in this animal model. None of the mice inoculated with the non-encapsulated Δ*cps14B* mutant strain died from the infection ($P < 0.0001$), and few clinical signs were observed. At 12 h after infection the mice inoculated with the wild-type strain had a mean blood bacterial burden of 2×10^8 CFU/mL, significantly greater ($P < 0.001$) than the burden of the mice inoculated with the Δ*cps14B* mutant (Figure 3B). At 24 h after infection the few surviving mice infected with the wild-type strain had a blood bacterial burden similar to that at 12 h and also significantly greater ($P < 0.001$) than that of the mice inoculated with the Δ*cps14B* mutant (data not shown). As Δ*cps14B* is avirulent in our infection model, the *in vivo* results confirm the phagocytosis results and demonstrate the crucial role of CPS in the virulence of *S. suis* serotype 14.

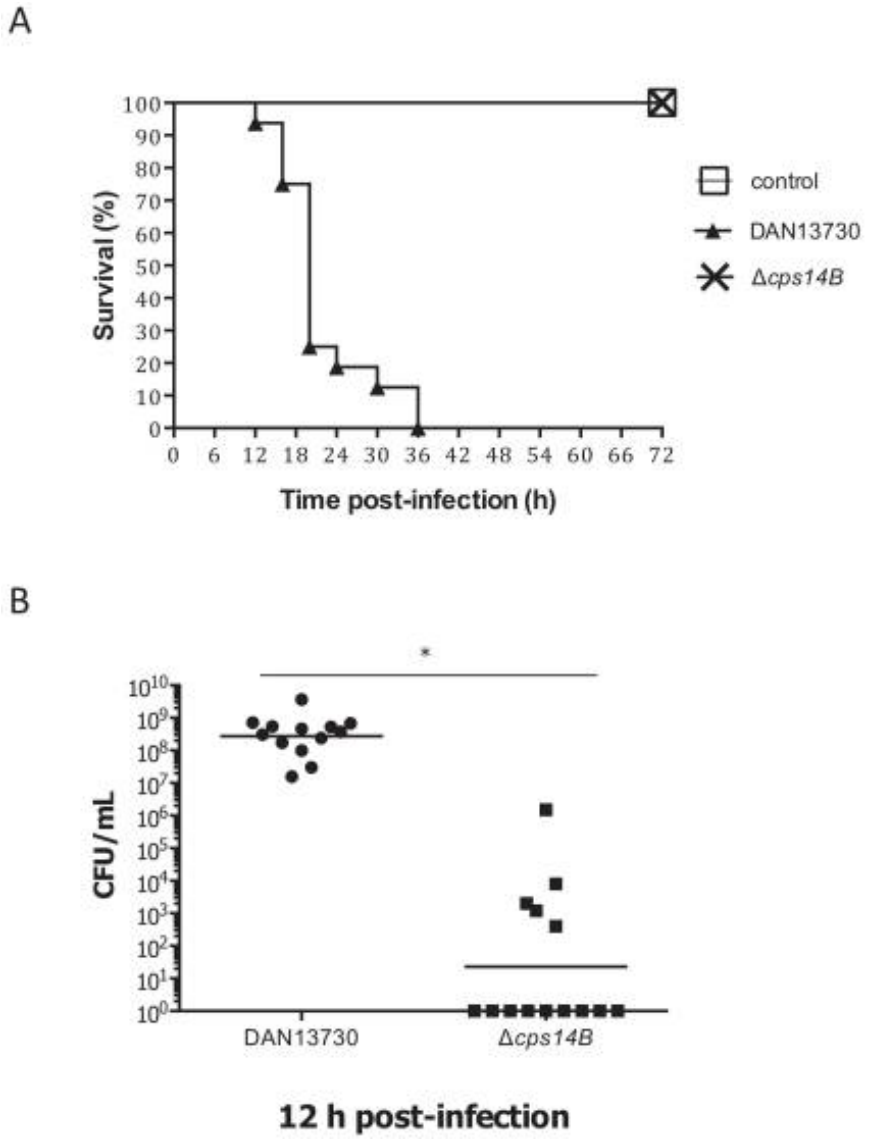

Figure 3. Survival (A) and blood bacterial burden (B) of CD1 mice inoculated by intraperitoneal injection with 1.5×10^8 CFU of DAN13730 or Δ*cps14B* or with Todd Hewitt broth as a control. Bacterial counts were determined in serial dilutions of 5 µL of blood obtained from the tail vein 12 h after inoculation. The Mantel–Cox log-rank test revealed a significant difference ($P < 0.0001$) in survival rate between the groups: all mice receiving the wild-type strain died, whereas all those receiving the mutant strain survived, and all the control animals were unaffected. The Mann–Whitney rank-sum test showed a significant difference ($P < 0.001$) in blood bacterial burden between the mice inoculated with the wild-type strain and those inoculated with the mutant strain (asterisk).

Conclusion

In conclusion, whereas there are differences in CPS composition and structure between serotypes 2 and 14, this study has demonstrated that the CPS of serotype 14 possesses important antiphagocytic properties and is a critical virulence factor. Since serotypes 14 and 1 have not only epitopes in common but also highly similar *cps* clusters and highly similar CPS structures (9; unpublished observations), the serotype 1 CPS may play a role in virulence and phagocytosis similar to that of serotypes 2 and 14. Further studies are needed to confirm this theory. This is the first report on the role of the CPS of an *S. suis* serotype other than 2. As with serotype 2, it has so far been impossible to evaluate the specific role of sialic acid in the virulence of *S. suis* serotype 14 since no CPS is produced in the absence of this sugar. Although the mouse model used in this study may be used to evaluate the systemic virulence of *S. suis* serotype 14, other models of meningitis with this serotype must be standardized. Further characterization and investigation will be necessary to dissect other virulence factors that would explain why serotype 2 strains are more widespread globally and seem to be more virulent in the field than serotype 14 strains.

Materials and Methods

The well-encapsulated *S. suis* serotype 14 reference strain DAN13730, isolated from a human case in The Netherlands (13), was used as the host strain for in-frame allelic deletion mutagenesis. The well-encapsulated virulent serotype 2 strain P1/7 and its previously obtained isogenic capsule-deficient mutant Δ*cps2F* (14) were used for comparison purposes. The bacterial strains and plasmids used in this study are listed in Table I. The *S. suis* strains were grown in Todd Hewitt broth (THB) or agar (THA) (Becton-Dickinson, Sparks, Maryland, USA) at 37°C. Precise in-frame deletions in *cps14B* and *neu14C (cps14Q)* were achieved by using splicing-by-overlap- extension polymerase chain reaction (PCR), as previously described (16).

Table I. Bacterial strains and plasmids used in this study

Strains/Plasmid/Primers	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 ΔlacX74 galU galK hsdR2*(rK- mK+) *mcrB1 rpsL*	[35]
Streptococcus suis		
P1/7	Wild-type, highly encapsulated serotype 2 strain isolated from a clinical swine case in the United Kingdom	[36]
DAN13730	Wild-type, highly encapsulated serotype 14 strain isolated from a human case in The Netherlands	[22]
Δ*cps2F*	Non-encapsulated isogenic mutant strain derived from strain P1/7. Deletion of the *cps2F* gene	[23]
Δ*cps14B*	Non-encapsulated isogenic mutant strain derived from strain DAN13730. Deletion of the *cps14B* gene	This work
Δ*neu14C*	Non-encapsulated isogenic mutant strain derived from DAN13730. Deletion of the *neu14C* gene.	This work
Δ*cps14B/cps14B*	Mutant Δ*cps14B* complemented with pMX14B complementation vector	This work
Δ*neu14C/neu14C*	Mutant Δ*neu14C* complemented with pMXNEU14C complementation vector	This work
Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Invitrogen
pSET4s	Thermosensitive vector for allelic replacement. Replication functions of pG+host3 and pUC19, MCS and *lacZ* of pUC19, Sp^R	[27]
pMX1	Replication functions of pSSU1, MCS of pUC19, Sp^R, malX promoter of *S. suis,* derivative of pSET2	[37]

p4Δcps14B	pSET4s carrying the construct for *cps14B* allelic replacement	This work
p4Δneu14C	pSET4s carrying the construct for *neu14C* allelic replacement	This work
pMX14B	pMX1 complementation vector carrying intact *cps14B*	This work
pMXNEU14C	pMX1 complementation vector carrying intact *neu14C*	This work

Genomic DNA of *S. suis* was purified by InstaGene Matrix solution (BioRad Laboratories, Hercules, California, USA). The primers used for the construction of deletion alleles (Table II) were obtained from Integrated DNA Technologies (Coralville, Iowa, USA). The PCR reactions were carried out with iProof proofreading DNA polymerase (BioRad Laboratories) or with *Taq* DNA polymerase (Qiagen, Valencia, California, USA). Amplification products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced with an ABI 310 automated DNA sequencer and the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA). Overlapping PCR products were cloned into plasmid pCR2.1 (Invitrogen, Burlington, Ontario), extracted with EcoRI, and recloned into the thermosensitive *Escherichia coli–S. suis* shuttle vector pSET4s digested with the same enzyme, which gave rise to the p4Δcps14B and p4Δneu14C mutation vectors (17). The recombinant plasmids were extracted and purified with the QIAprep Spin Miniprep kit (Qiagen). Restriction enzymes and DNA-modifying enzymes were purchased from Fisher Scientific (Ottawa, Ontario) and used according to manufacturer recommendations.

Table II. Oligonucleotide primers used in this study

Oligonucleotide primers, sequence (5' – 3').		Constructs
cps14B-ID1	GACCAAGATAACATCACCGC	p4Δcps14B
cps14B-ID2	GGTTGGCATTTGTCTACAGT	p4Δcps14B
cps14B-ID3	ACCACTCCAAATACAAAACG	p4Δcps14B
cps14B-ID4	GCTCGCGCTATATTCTCTTG	p4Δcps14B
cps14B-ID5	CGACTTGGTGGGTGGAATTG	p4Δcps14B
cps14B-ID6	TCTTCGATGTCCTGAGGACGGTCAACACTGCAGTTAAGAG	p4Δcps14B
cps14B-ID7	CTCTTAACTGCAGTGTTGACCGTCCTCAGGACATCGAAGA	p4Δcps14B
cps14B-ID8	GGTTTCTCCCAACCCTACTG	p4Δcps14B
neu14C-ID1	CGGTGATGTTCATCTAGCACGG	p4Δneu14C
neu14C -ID2	AGCGATCCCCCAGAATCAACAC	p4Δneu14C
neu14C -ID3	CACAGCCGAAGAACAAACGCAG	p4Δneu14C
neu14C -ID4	TGGACGCATGAGGACTTGAACC	p4Δneu14C
neu14C -ID5	TCTCAGCTCGAAATGACTCGTC	p4Δneu14C
neu14C -ID6	CATGGTTGAGGCCTGACGAGAGCCTGTCAC	p4Δneu14C
neu14C -ID7	GTGACAGGCTCTCGTCAGGCCTCAACCATG	p4Δneu14C
neu14C -ID8	AGGTCCCTGACTCCGTCAAC	p4Δneu14C
pCPS14BF_NcoI[b]	AGCCATGGAGTCCGTACTTGTTTA	pMX14B
pCPS14BR_EcoRI[b]	GTACGTGGAATTCCTAACATTGCC	pMX14B
pNEU14CF_PstI[b]	TGAGCTGCAGCAAAATATTTGCCATAGTGC	pMXNEU14C
pNEU14CR_PstI[b]	CATCTGCAGAGGTACCCGCTCCTAGAAAGG	pMXNEU14C

Final constructions of pSET4s, p4Δcps14B, and p4Δneu14C were electroporated into *S. suis*-competent cells with the Biorad Gene PulserXcell apparatus (BioRad Laboratories) under specific conditions: 12.5 kV/cm, 200 Ω, and 25 μF. Transformants were plated on THA supplemented with spectinomycin (THA + Sp) and incubated for 3 d at 28°C. Several Sp-resistant colonies were then subcultured on THA + Sp for 3 d at 28°C. The candidates were next cultured on THA + Sp and incubated at 37°C for 2 successive passages. Temperature- and Sp-resistant clones were successively cultured on THA and THA + Sp to obtain Sp-sensitive candidates. Deletion of the genes *cps14B* and *neu14C* was confirmed by PCR and sequence analysis.

For complemented mutants, intact *cps14B* and *neu14C* genes were amplified from genomic DNA of the wild-type strain with primers designed with restriction sites (Table II).

The PCR products and pMX1 vectors were then digested with the appropriate restriction enzyme before ligation. Final constructions were cloned into *E. coli* MC1061. The *E. coli* strains were grown in Luria–Bertani broth or agar (Becton-Dickinson) at 37°C. When needed, antibiotics (Sigma-Aldrich Canada, Oakville, Ontario) were added to the culture medium at the following concentrations: for *S. suis,* spectinomycin at 100 μg/mL; for *E. coli,* kanamycin and spectinomycin at 50 μg/mL; and for *E. coli,* ampicillin at 100 μg/mL. Complementation of both mutants was achieved by electroporation with pMX14B and pMXNEU14C under the conditions mentioned previously.

Serotyping was carried out by coagglutination tests as described by Gottschalk et al (13), and positive results were recorded when a strong reaction was obtained within 1 min. Transmission electron microscopy (TEM) was carried out as described by Jacques et al (18) with a few modifications. 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. Ferritin was then added, to a final concentration of 1 mg/mL, and the suspension incubated for 30 min at room temperature. Afterwards the cells were immobilized in 3% (w/v) agar, washed 5 times in cacodylate buffer containing 0.05% ruthenium red, and fixed with 2% (v/v) osmium tetroxide for 2 h. Samples were washed and dehydrated in graded series of acetone, then washed twice in propylene oxide and embedded in Spurr low-viscosity resin (Sigma-Aldrich Canada). Thin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (JEM 1230; JEOL, Tokyo, Japan) at 80 kV.

The J774A.1 murine macrophage-like cell line (American Type Culture Collection TIB 67; Rockville, Maryland, USA) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin, 25 units/mL (Gibco, Burlington, Ontario). The cells were grown at 37°C with 5% CO_2 until confluent. The cultures were then scraped and the cells washed twice with phosphate-buffered saline (PBS), pH 7.4, resuspended in antibiotic-free medium at 1×10^5 cells per well in a 24-well tissue culture plate (VWR CanLab, Montreal, Quebec), and incubated for 3 h at 37°C with 5% CO_2 to allow cell adhesion. The cells were infected by removing the medium and

adding 1 mL of a bacterial suspension, 1×10^7 colony-forming units (CFU)/mL in antibiotic-free medium; the multiplicity of infection (MOI) was thus 100. The infected cells were incubated for 60 min at 37°C with 5% CO_2 to allow phagocytosis. The optimal incubation time and MOI were chosen according to the results of preliminary studies (data not shown). After incubation, cell monolayers were washed twice with PBS and incubated for 1 h with medium containing penicillin G (Sigma-Aldrich Canada), 5 μg/mL, and gentamicin (Gibco), 100 μg/mL, to kill extracellular bacteria. The cell monolayers were washed 3 times with PBS and lysed with sterile water. The presence of viable intracellular bacteria was determined by plating serial dilutions on THA. Each test was repeated 4 times in independent experiments.

A well-standardized *S. suis* serotype 2 murine model of infection (19) was adapted for the first time to serotype 14. Six-week-old female CD1 mice (Charles River Laboratories, Wilmington, Massachusetts, USA) were used, the experiments involving them being 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. The 45 animals were divided into 3 groups of 15 animals each. On the day of the experiment, each animal was inoculated by intraperitoneal injection with 1.5×10^8 CFU of the *S. suis* serotype 14 wild-type strain DAN13730 or the mutant strain Δ*cps14B* or with a vehicle (THB) as a control. The bacterial concentration was determined from preliminary trials with DAN13730 to establish a high but controlled mortality level (data not shown). The mice were examined at least 3 times daily for clinical signs of septic disease, such as depression, swollen eyes, rough coat hair, and lethargy, for 72 h after infection. Blood samples (5 μL) were collected from the tail vein at 12, 24, 48, and 72 h after infection and plated on THA for evaluation of bacteremia.

All data are expressed as mean ± standard error. For the *in vitro* experiments, data were analyzed for significance with the Mann–Whitney rank-sum test. For the *in vivo* virulence experiments, the Mantel–Cox log-rank test was used to evaluate the difference in mortality rate between the groups, and the Mann–Whitney rank-sum test was used to evaluate the difference in bacteremia between the groups. A *P*-value of less than 0.05 was considered significant.

Acknowledgments

We thank Sonia Lacouture for her assistance. This work was supported by Natural Sciences and Engineering Research Council of Canada grant 154280 to Dr. Gottschalk and grant 342150-07 to Dr. Segura and partially by Grant-in-Aid for Young Scientists (B) (23480310) to Dr. Okura from the Japan Society for the Promotion of Science.

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ARTICLE III

A single amino acid polymorphism in the glycosyltransferase CpsK defines four *Streptococcus suis* serotypes

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Publié dans Scientific Report. **2017.** doi: 10.1038/s41598-017-04403-3

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 et à la réalisation des expériences, à l'analyse des résultats et à l'écriture de l'article.

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).

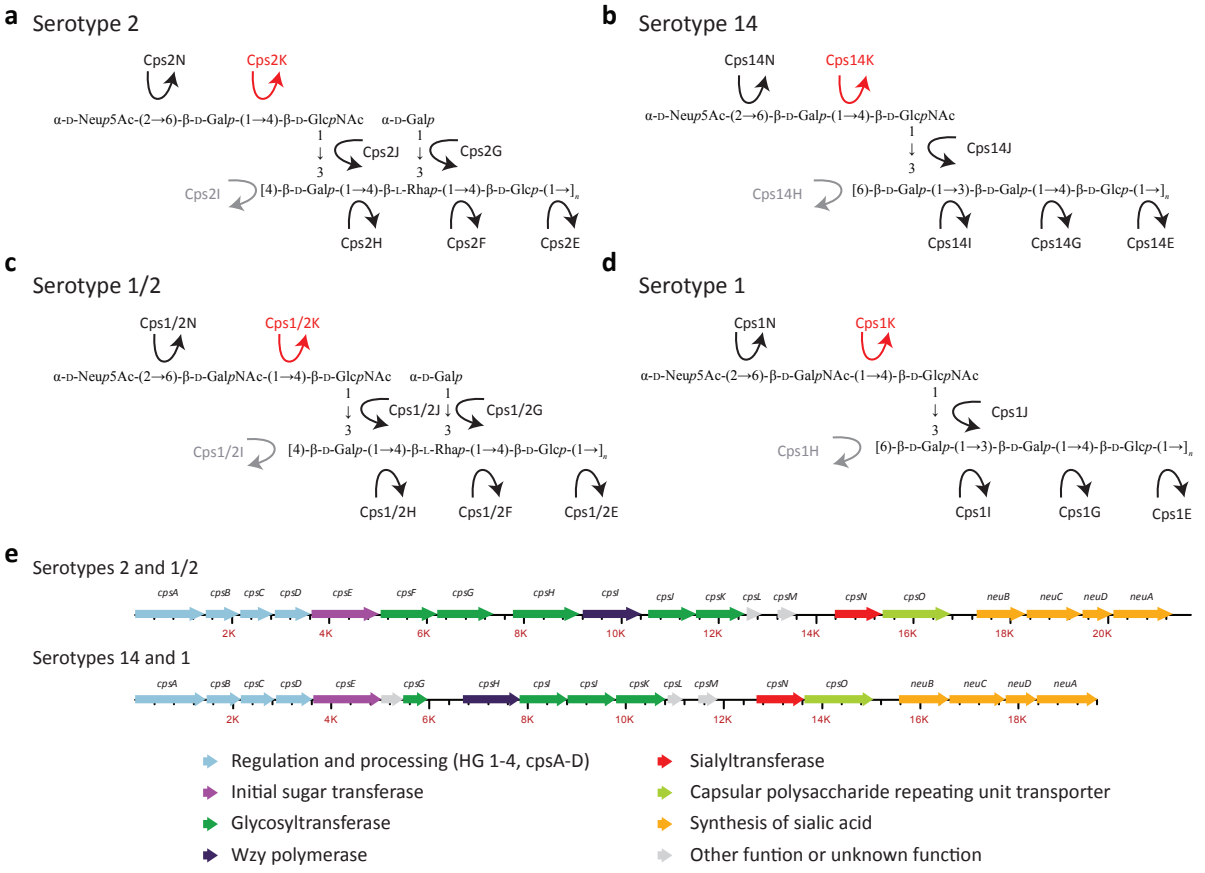


Figure 1. Capsular polysaccharide structures of *S. suis* serotypes 2, 1/2, 14, and 1 capsular polysaccharides, and schematics of the *cps* loci of these serotypes. a–d, CPS structures of serotype 2 (a), serotype 14 (b), serotype 1/2 (c), and serotype 1 (d). In serotypes 1 and 1/2, CpsK is predicted 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.

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].

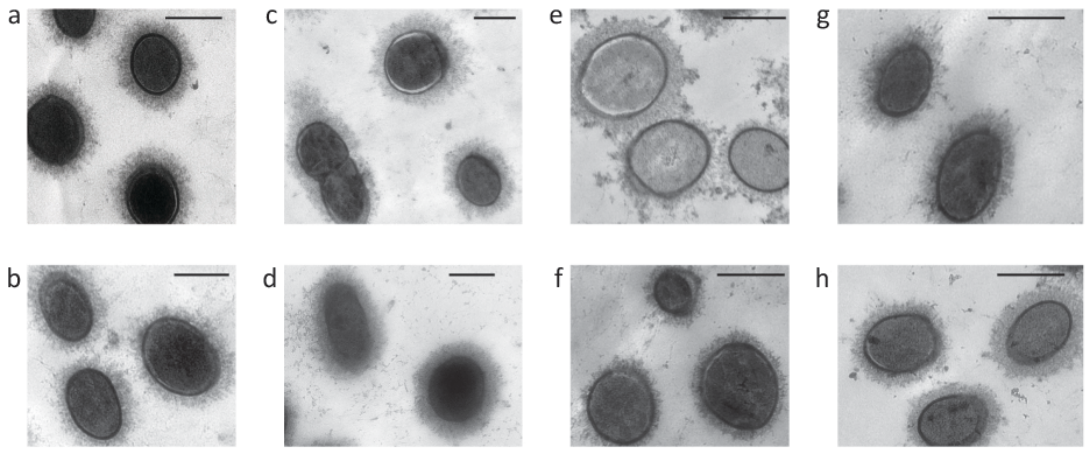
Here, we tested the hypothesis that this single amino acid polymorphism is the key factor influencing the sugar residue (Gal or GalNAc) added to the CPS repeating unit by either CpsK variants. We show that polymorphic CpsK variants define expression by *S. suis* strains of either serotype 2 or 1/2 CPSs, 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.

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).



Supplementary Figure 1. Transmission electron micrographs showing capsular polysaccharide (CPS) expression by *S. suis* field strains and derivative capsular switch mutants. The CPS was stabilized with polyclonal antibodies directed against the CPS (see methods). a) Serotype 2 field strain. b) Mutant strain SS2to 1/2. c) Serotype 1/2 field strain. d) Mutant strain SS1/2to2. e) Serotype 14 field strain. f) Mutant strain SS14to1. g) Serotype 1 field strain. h) Mutant strain SS1to14. No noticeable differences were observed between field strains and corresponding derivative mutant strains in CPS expression. Bar: 500 nm.

Supplementary Table I. Average yields for CPS purification of the different *S. suis* field and mutant strains and CPS molecular weights.

Strain	Serotype	Average yield ± SD (mg/6 L)	M_w (kg/mol)	Reference
SS2	2	46.3 ± 19.1 (n=13) [a]	435 ± 32.1 (n=4)[a]	[17]
SS2to1/2	1/2	51.0 (n=1)	483 (n=1)	This work
SS1/2	1/2	73.5 ± 2.1 (n=2)	709 (n=1)	[19]
SS1/2to2	2	44.6 (n=1)	504 (n=1)	This work
SS14	14	30.1 ± 14.8 (n=11) [a]	421 ± 117 (n=3) [a]	[18]
SS14to1	1	28.2 (n=1)	490 (n=1)	This work
SS1	1	37.5 ± 9.2 (n=2)	741 (n=1)	[19]
SS1to14	14	32.4 (n=1)	571 (n=1)	This work

[a] Means calculated from published and unpublished laboratory data.

Table I. 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

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).

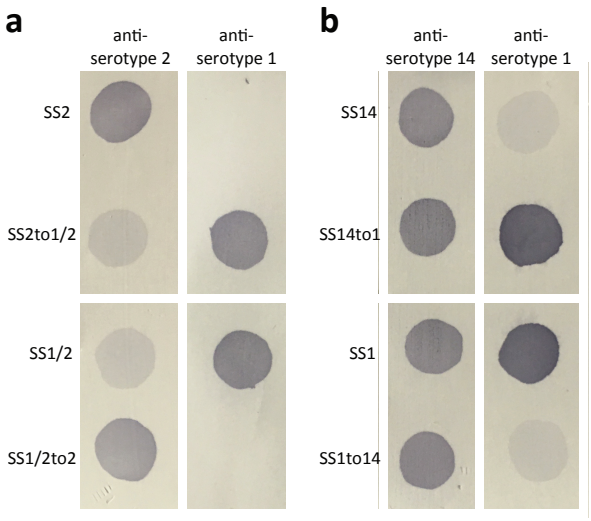


Figure 2. Serotype switching of mutants is confirmed by dot blotting of purified CPS preparations and specific antisera. a, the CPS from a serotype 2 field strain reacts with anti-serotype 2 but not with anti-serotype 1 sera, while the CPS from mutant strain SS2to1/2 (W161C) reacts with both antisera (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).

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).

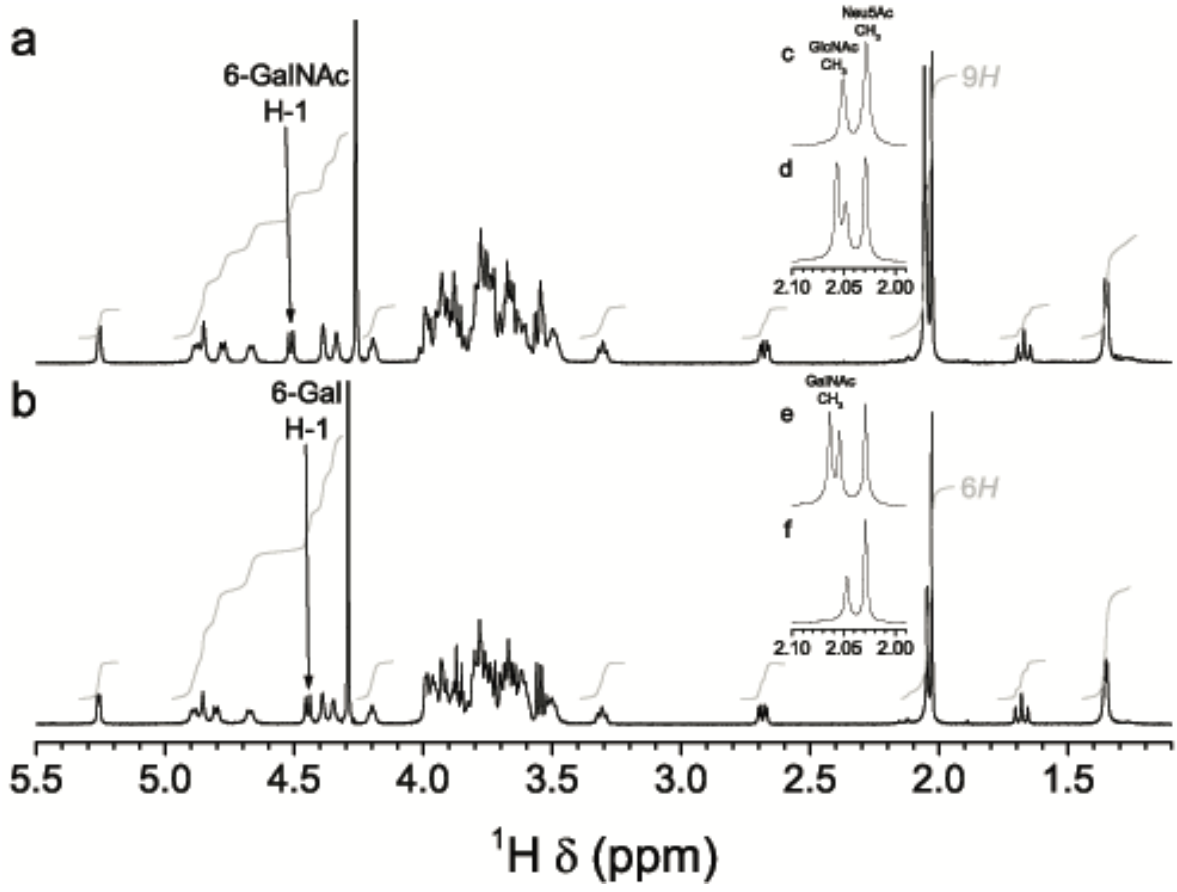


Figure 3. 1D ^{1}H NMR spectra of CPS preparations from serotypes 2 and 1/2 in 33 mM phosphate pD 8.0 in D_2O. a–b, Full spectrum. c–f, Expansion of the methyl region. a, d, SS2to1/2 mutant, 500 MHz, 77°C. b, f, SS1/2to2 mutant, 500 MHz, 75°C. c, Serotype 2 field strain, 600 MHz, 50°C [17]. e, Serotype 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.

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).

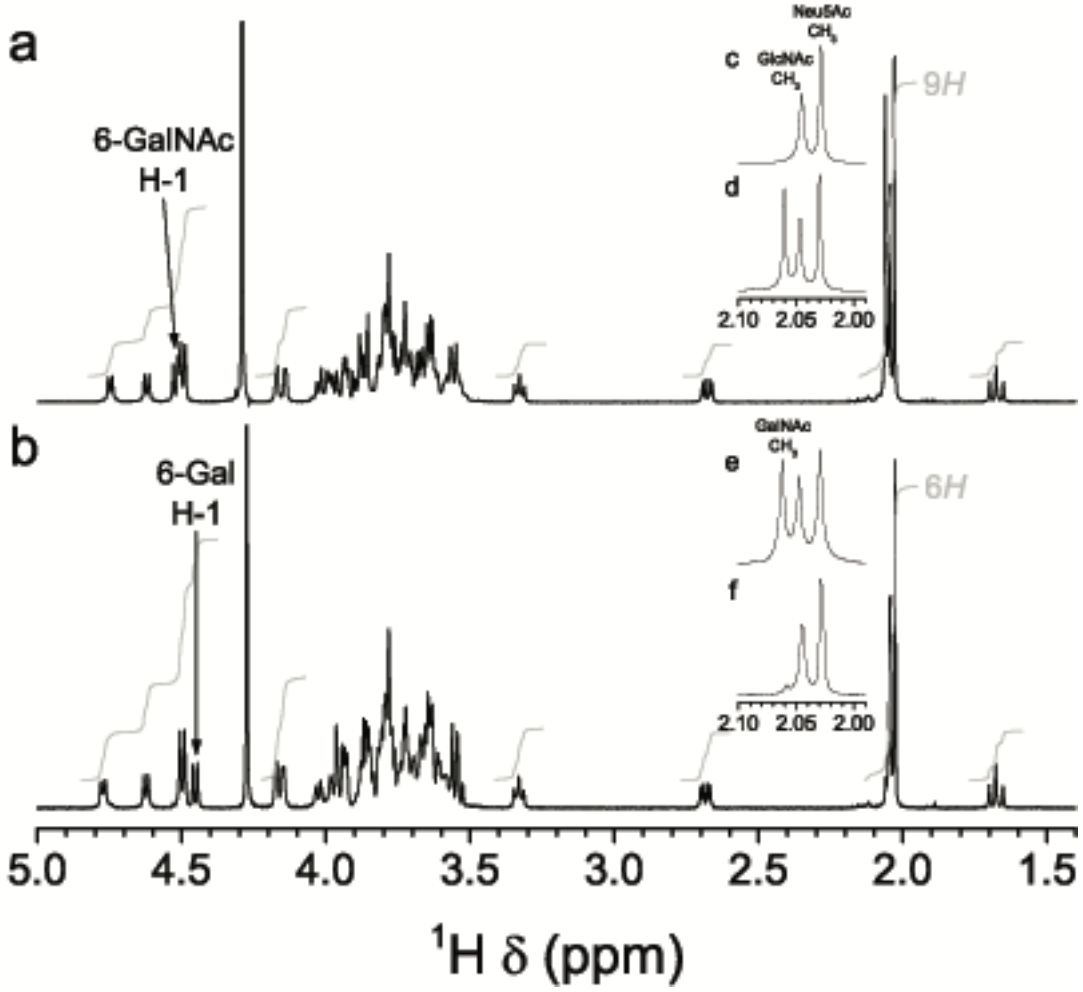
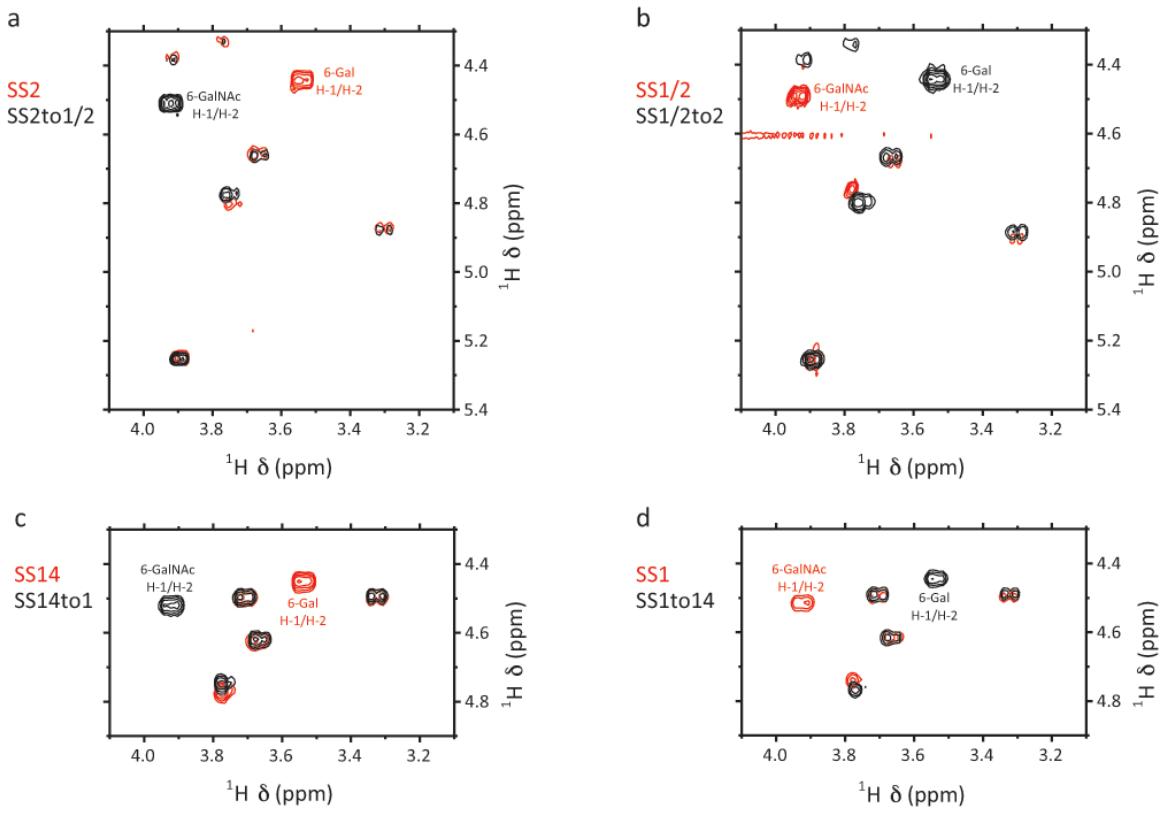


Figure 4. 1D ^{1}H NMR spectra of CPS preparations from serotypes 14 and 1 in 33 mM phosphate pD 8.0 in D_2O. a–b, Full spectrum. c–f, Expansion of the methyl region. a, d, SS14to1 mutant, 500 MHz, 75°C. b, f, SS1to14 mutant, 500 MHz, 77°C. c, Serotype 14 field strain, 500 MHz, 77°C [18]. e, Serotype 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.

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.

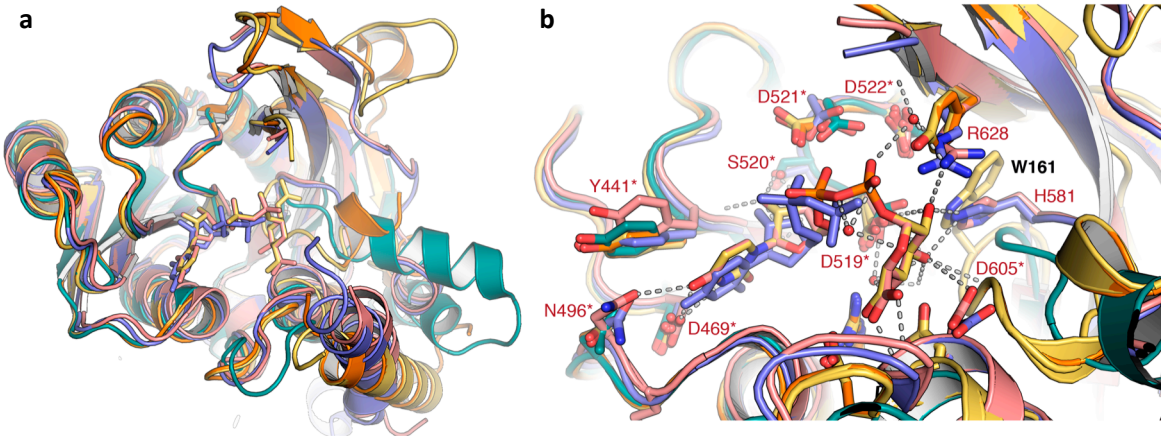


Supplementary Figure 2. Portion of the 500 MHz ge 2D NMR COSY spectrum of *S. suis* CPSs in 33 mM phosphate pD 8.0 in D2O. a) The SS2to1/2 mutant is depicted in black. 77°C. 512 increments of 1 K complex data points were acquired in magnitude mode with a digital resolution of 3.9 Hz/point in the t2 dimension and 7.8 Hz/point in the t1 dimension; the t2 dimension was processed by multiplication with an unshifted sine bell window function and Fourier transform, and the t1 dimension was processed by Zhu-Bax linear prediction to 1024 points, multiplication with an unshifted sine bell window function, Fourier transform, and magnitude calculation. The serotype 2 field strain is depicted in red. 75°C. 512 increments of 1 K complex data points were acquired in magnitude mode with a digital resolution of 4.6 Hz/point in the t2 dimension and 9.2 Hz/point in the t1 dimension. Processing was as above. b) Mutant SS1/2to2 (black). 75 °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 t2 dimension and 9.8 Hz/point in the t1 dimension; processing was as described in a. Serotype 1/2 field strain (red), 700 MHz, 42°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 t2 dimension and 13.7 Hz/point in the t1 dimension. Processing was as described in a. c) Mutant strain SS14to1 (black), 75 °C. Serotype 14 field strain (red), 77°C. Acquisition and processing were as described in b. d) Mutant strain SS1to14, 77°C. Serotype 1 field strain, 65°C. Acquisition and processing were as described in b.

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 chain 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 *M*w 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).



Supplementary Figure 3. Structural and substrate-binding conservation in closest homologues of CpsK from *S. suis*. a) Structural superposition of the model of CpsK from *S. suis* in complex with UDP-GalNAc (yellow ribbon and sticks) and the putative glycosyltransferase (GalT1) from *Streptococcus parasanguinis* (PDB code 5hec, orange ribbon), the chondroitin polymerase from *Escherichia coli* strain K4 in complex with UDP (PDB code 2z87, blue ribbon), the chondroitin polymerase from *Escherichia coli* strain K4 complexed with UDP-GlcUA (PDB code 2z86, pink ribbon) and the glycosyltransferase from *Bacteroides fragilis* (PDB code 3bcv, green ribbon). All these structures share a common fold and the position of many key residues that interact with the dinucleotide with virtually overlaps within the structures. The estimated RMSD value for the Cα backbone of the catalytic core between all the structures was less than 1.14 Å. B) Detailed view of the nucleotide-binding site in CpsK serotype 2 (yellow ribbon) in complex with UDP-GalNAc (yellow sticks) and its closest homologues (color code as in panel A). Residues involved in substrate binding are represented as capped sticks. Interactions between chondroitin polymerase from *Escherichia coli* strain K4 and UDP-GlcUA (PDB code 2z86) are represented as dashed lines. Residues involved in the interaction are labeled. Asterisk indicates if the residue is conserved in CpsK. Trp161 in CpsK is depicted as yellow capped sticks to stress potential conservation of the interaction with O2 of Gal ring observed for His581 with glucuronic acid.

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).

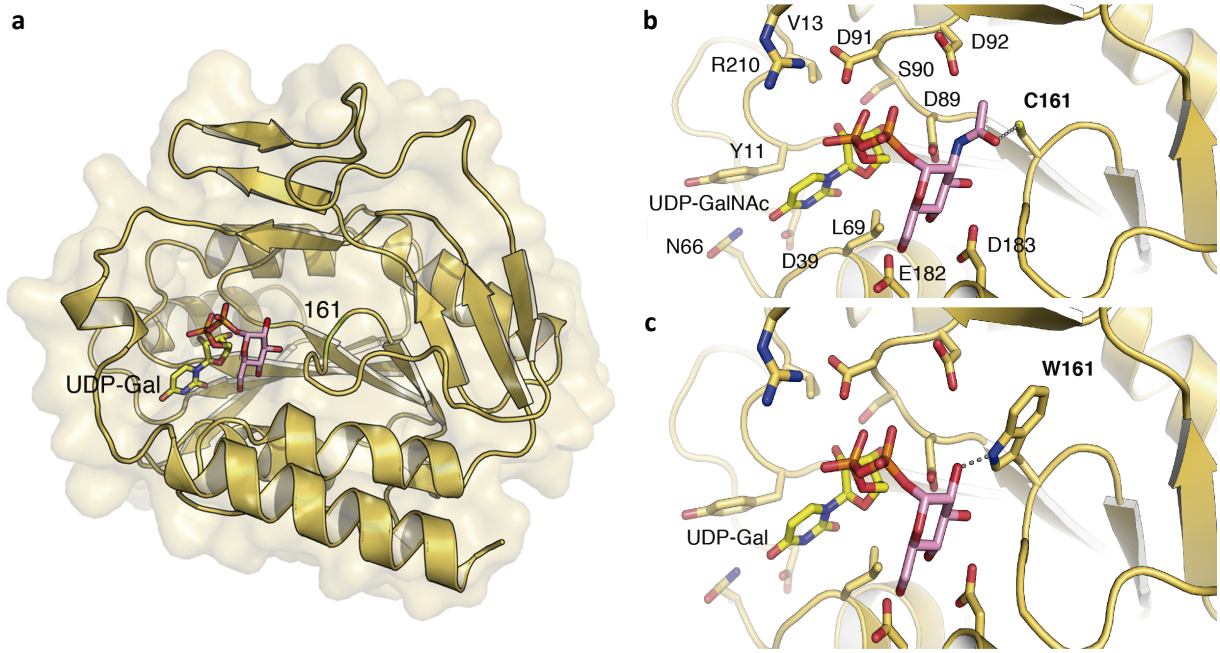
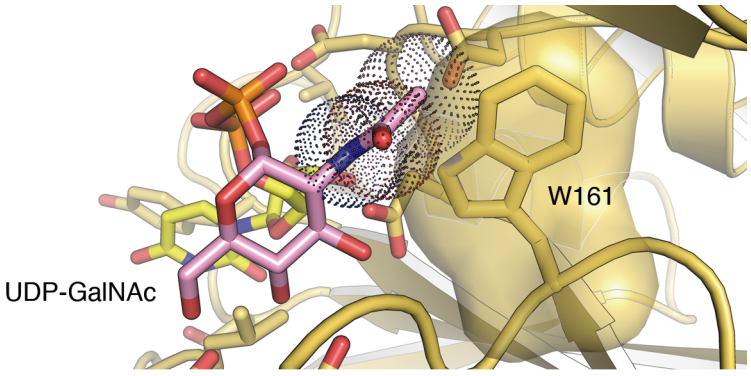


Figure 5. Three-Dimensional modeling of CpsK polymorphic variants. a, The serotype 2 CpsK protein structure is depicted in yellow ribbon, and the position of amino acid residue 161 was colored in green and labeled. The docked substrate UDP-Gal is shown in sticks with the Gal moiety in pink. b, Detailed 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.

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.



Supplementary Figure 4. Steric hindrance between tryptophan 161 and the N-acetyl group of UDP-GalNAc. Tryptophan residue is labeled and depicted in sticks with its molecular surface shown in yellow. The Van der Waals radius of the N-acetyl group is shown as dots representation. The presence of bulky Trp residue at position 161 would prevent binding of GalNAc at the active site of CpsK from serotype 2.

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.

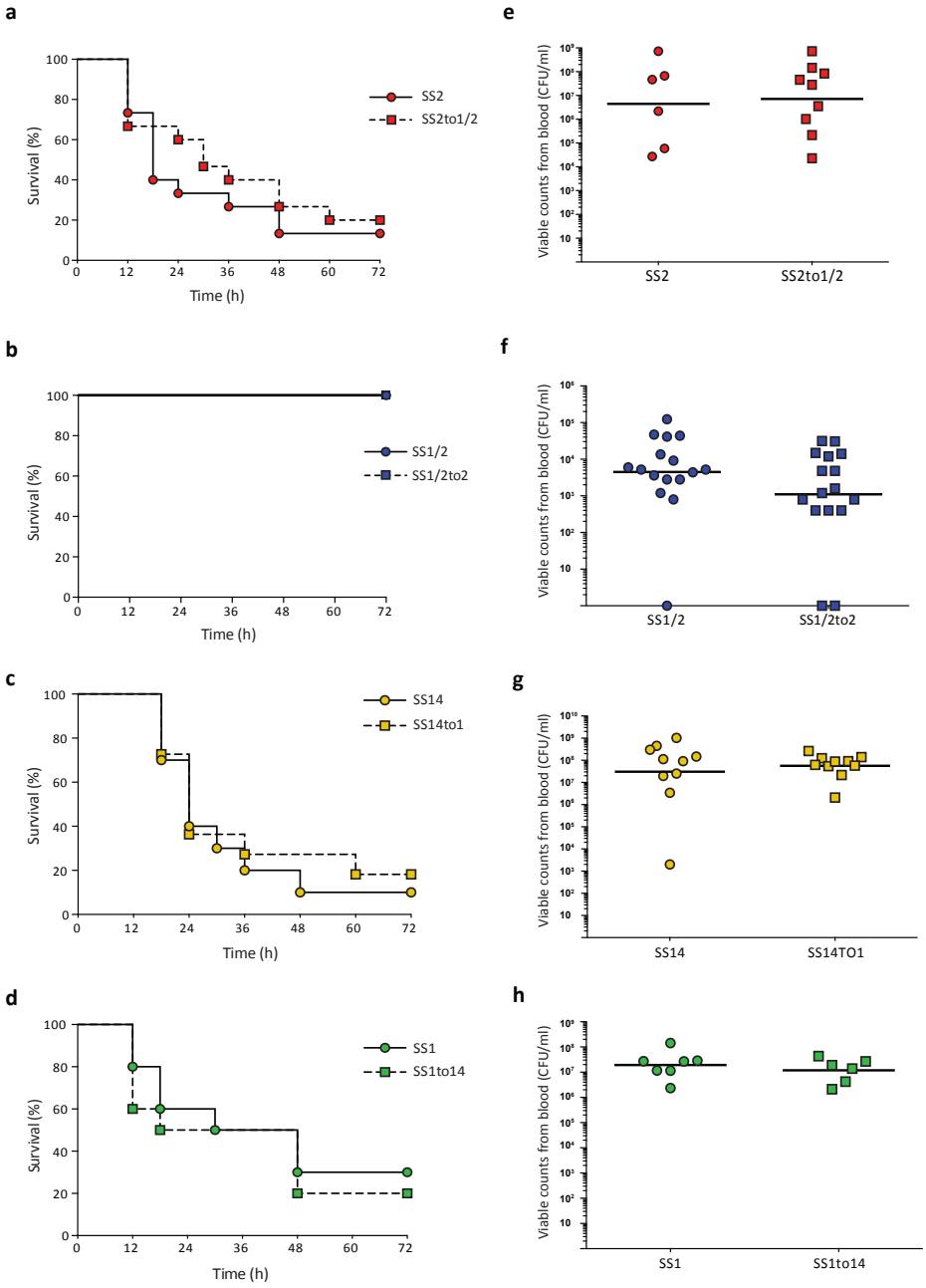


Figure 6. Serotype switching does not impact strain virulence. a–d, Survival of CD1 mice inoculated by intraperitoneal injection with either 5×10^7 CFU of serotype 2 (a) or 1/2 (b) strains or with either 1×10^8 CFU of serotype 14 (c) or 1 (d) strains. In all cases, Log-Rank (Mantel-Cox) test revealed 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, $P<0.05$).

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.

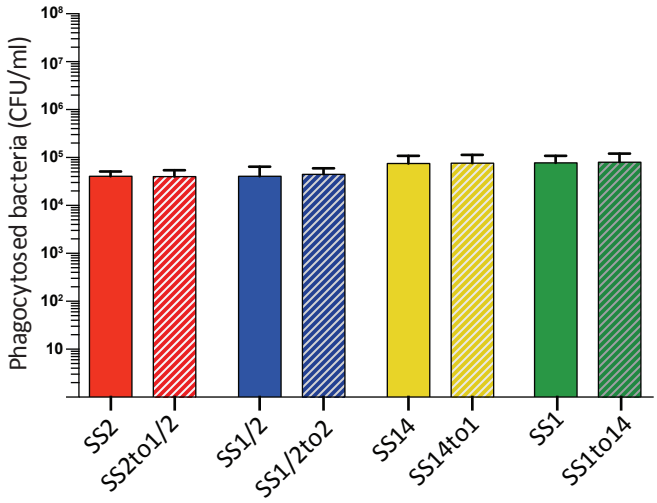


Figure 7. The antiphagocytic properties afforded to *S. suis* by CPS types 2, 1/2, 14, and 1 appear similar under *in vitro* conditions. Parental strains and isogenic mutants (1×10^7 CFU/ml) were incubated for 60 min with J774 macrophages (multiplicity of infection = 100) in the presence of 50% murine 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.

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.

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.

Supplementary Table II. Bacterial strains and plasmids used in this study

Strains/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	ThermoFisher
Streptococcus suis		
SS2	Strain P17; ST1 encapsulated serotype 2 strain isolated from a swine clinical case of infection in the United Kingdom. Identified as SS2 in this study.	[4]
SS1/2	Strain 2651; ST28 encapsulated serotype 1/2 strain isolated from a swine clinical case of infection in Denmark. Identified as SS1/2 in this study.	[5]
SS14	Strain DAN13730; ST6 encapsulated serotype 14 strain isolated from a human case in The Netherlands. Identified as SS14 in this study.	[6]
SS1	Strain 1659834; ST1 encapsulated serotype 1 strain isolated from a swine clinical case of infection in	This work

	Canada. Identified as SS1 in this study.	
SS2to1/2	Isoallelic *cpsK* mutant of serotype 2 strain P1/7 carrying G483T mutation predicted to result in W161C substitution in mature CpsK	This work
SS1/2to2	Isoallelic *cpsK* mutant of serotype 1/2 strain 2651 carrying T483G mutation predicted to result in C161W substitution in mature CpsK	This work
SS14to1	Isoallelic *cpsK* mutant of serotype 14 strain DAN13730 carrying G483T mutation predicted to result in W161C substitution in mature CpsK.	This work
SS1to14	Isoallelic *cpsK* mutant of serotype 1 strain 1659834 carrying T483G mutation predicted to result in C161W substitution in mature CpsK.	This work
Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	ThermoFisher
pSET-4s	Thermosensitive vector for allelic replacement. Replication functions of pG+host3, MCS *oriR* pUC19 *lacZ* Sp^R	[7]
p4cpskG483T	pSET-4s carrying the construct for allelic replacement in strain P1/7 (SS2) and strain DAN13730 (SS14)	This work
p4cpskT483G	pSET-4s carrying the construct for allelic replacement in strain 2651 (SS1/2) and strain 1659834 (SS1)	This work

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).

Supplementary Table III. Oligonucleotide primers used in this study**.**

Name	Sequence (5' – 3')	Constructs
sero1-2_ID1	GCGGTATCTTTAATAGCCCTTG	p4cpskT483G
sero14_ID1	AGATACTATACGTTGGCAAG	p4cpskG483T
sero1-2_ID4	CATAGTAACTCCCAACTCCCTG	p4cpskT483G, p4cpskG483T
sero14_ID5	GAGATTCTTCTGGTGAATGACG	p4cpskT483G, p4cpskG483T
sero1-2_ID8	CCCCGTTTTCAGAAAGACAC	p4cpskT483G, p4cpskG483T

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.

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 an scholarship from the Swine and Poultry Infectious Disease Research Centre.

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ARTICLE IV

Streptococcus suis sialyltransferase exhibits alpha2-6 activity in Group B *Streptococcus*

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Article soumis dans: Frontiers in Microbiology

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 et à la réalisation des expériences, à l'analyse des résultats et à l'écriture de l'article.

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 biochemical characteristics and similarities in the *cps* loci, sialic acid exerts differential control of CPS expression by *S. suis* and GBS.

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 colonisation - the first step of the infection – and, more importantly, during invasion and dissemination within the host. *Streptococcus suis* and Group B *Streptococcus* (GBS) are two well-encapsulated Gram-positive bacteria that were extensively studied in the last years/decades due to their 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 (Cieslewicz et al., 2005; Johri et al., 2006; Goyette-Desjardins et al., 2014).

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* are meningitis and septicemia with sudden death. In humans, 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 (Goyette-Desjardins et al., 2014). On the other hand, GBS is an important cause of severe invasive bacterial infections worldwide (Johri et al., 2006; Madzivhandila et al., 2011). 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 (Johri et al., 2006; Madzivhandila et al., 2011).

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 (Cieslewicz

et al., 2005; Okura et al., 2013). 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 (Wessels et al., 1987; Wessels and Kasper, 1990; Wessels et al., 1991) and of *S. suis* types 2 and 14 (Van Calsteren et al., 2010; Van Calsteren et al., 2013) 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 (Charland et al., 1998; Chabot-Roy et al., 2006; Lecours et al., 2011a; Lecours et al., 2011b). 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 (Segura et al., 1998; Segura, 2012; Lemire et al., 2014).

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 (Van Calsteren et al., 2010; Okura et al., 2013; Van Calsteren et al., 2013). 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 (Bax et al., 2011). 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 sialyled 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.

Results

Transcription of 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 1**). 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 1**).

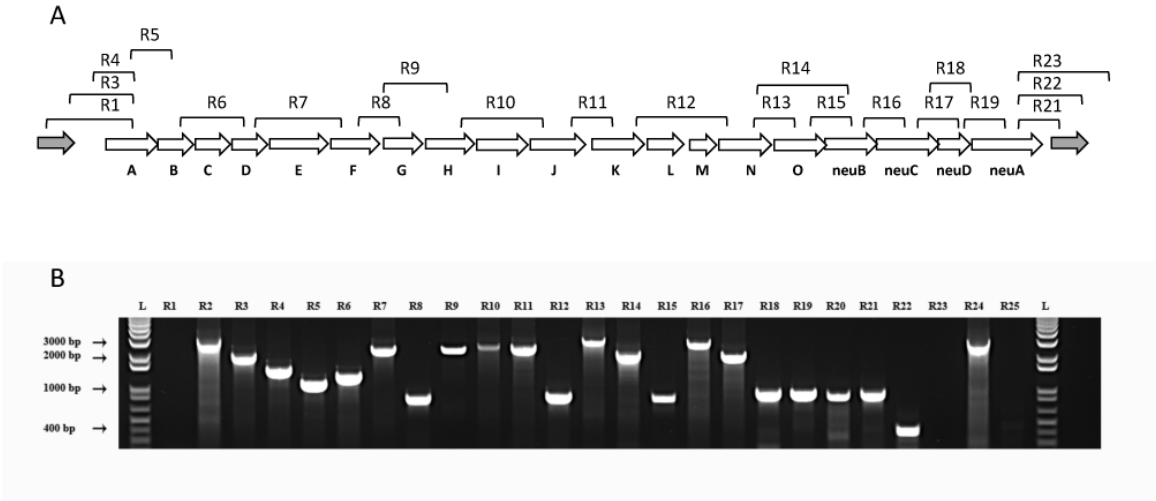
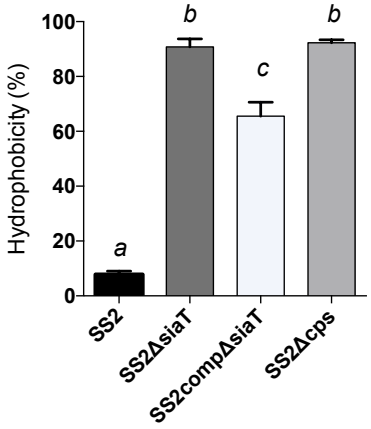


Figure 1. Transcriptional analysis of *S. suis cps* operon. (A) Schematic representation of *S. suis* CPS synthesis coding region with corresponding RT-PCR reactions and **(B)** agarose gel (1%) electrophoresis of RT-PCR reaction products visualized under UV light. Total bacterial RNA was isolated and converted to cDNA. RNA samples without reverse-transcription step was used as template to verify absence of genomic DNA in sample (lane R25). Genomic DNA with the same primers was used for positive control (lane R24). All RT-PCR products migrate accordingly to their expected sizes. DNA size standards are depicted at left.

Deletion of sialyltransferase in S. suis *results in non-encapsulated phenotype*

It has been previously reported that deletion of the *neuC* gene (sialic acid synthase) 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* (Lakkitjaroen et al., 2014), 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 (Lakkitjaroen et al., 2014). We thus took advantage of this particularity in order to knockdown the sialyltransferase gene in *S. suis* serotype 2 (mutant SS2ΔsiaT; see experimental procedures). As depicted in **Figure 2A**, 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, episomal complementation in *S. suis* with pMX1 results in an intermediate state when compared to deficient mutant and wild-type strain (Lecours et al., 2012; Roy et al., 2015; Ferrando et al., 2017). Absence of CPS expression by the SS2ΔsiaT mutant was also confirmed by TEM, as depicted in **Figure 2B**, where a total loss of CPS expression was observed in the mutant strain compared to an apparent thick CPS surrounding the wild-type strain.

A



B

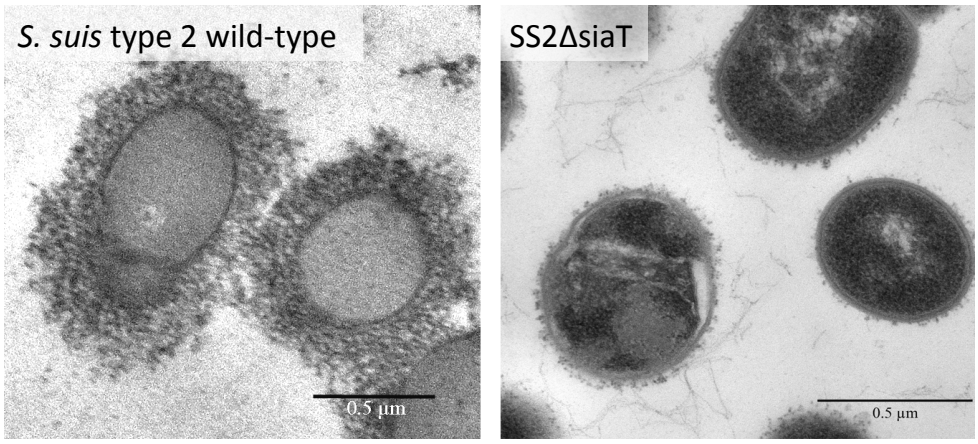


Figure 2. Capsular polysaccharide (CPS) expression levels of *S. suis* serotype 2 and derived isogenic mutants. (A) Hydrophobicity (%) of the wild-type *S. suis* serotype 2 strain (SS2), the sialyltransferase SS2ΔsiaT (Δ*cps2N*) mutant, the complemented SS2compΔsiaT mutant, and of the non-encapsulated 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.

Substitution of α2-6 sialyltransferase by GBS α2-3 sialyltransferase in* S. suis *serotypes 2 and 14 also results in a non-encapsulated phenotype

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 3A**). 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 3B** and **3C**, *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 4**).

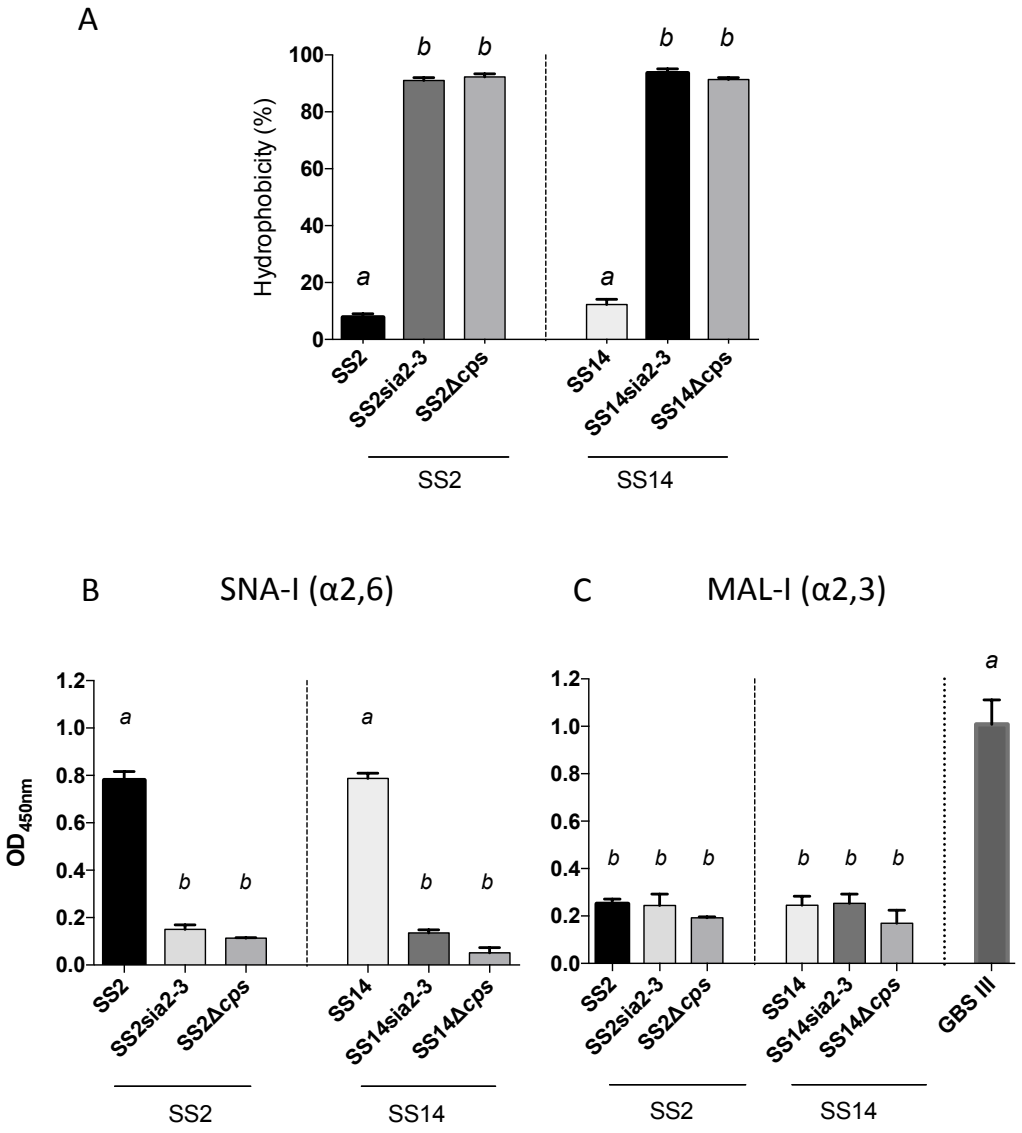


Figure 3. Capsular polysaccharide (CPS) expression levels and sialic acid linkage in *S. suis* serotype 2 and 14 mutants carrying exogenous α-2,3,sialyltransferase. (A) Hydrophobicity (%) of the wild-type *S. suis* serotypes 2 (SS2) and 14 (SS14) strains, the SS2sia2,3 (Δ*cps2N*/*cpsK*) and SS14sia2,3 (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' ($P < 0.05$).

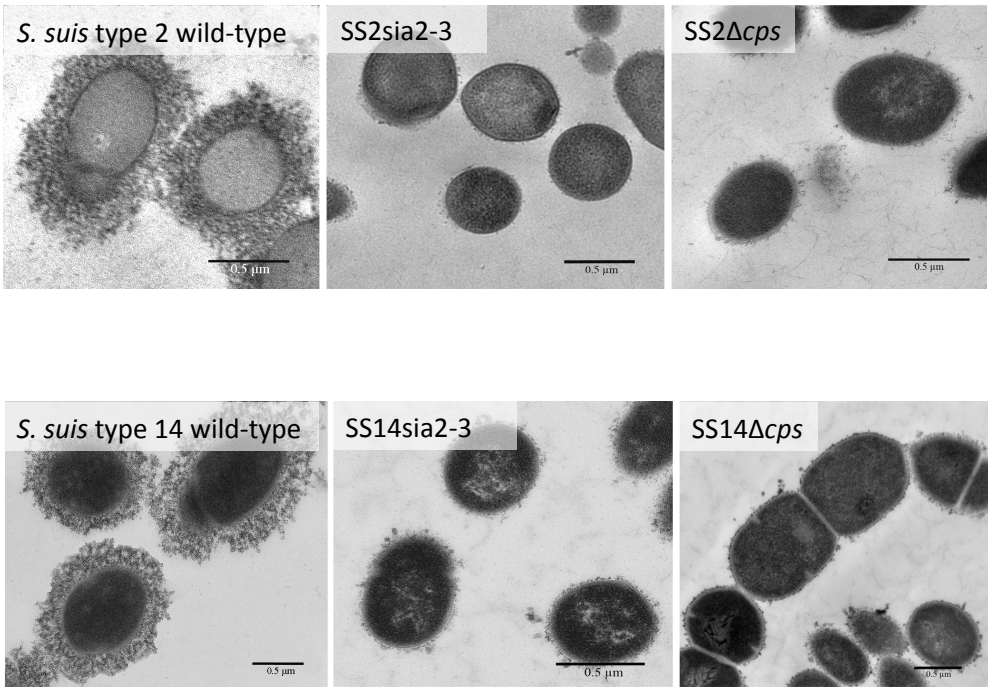


Figure 4. Transmission electron micrographs showing capsular polysaccharide (CPS) expression by *S. suis* serotype 2 and 14 isogenic mutants. The CPS was labelled with polycationic ferritin. *S. suis* serotype 2 wild-type strain and *S. suis* serotype 14 wild-type strain were surrounded by a thick capsule, 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.

Deletion of sialyltransferase or sialic acid synthase genes in GBS type V results in asialo phenotype

In order to determine if the non-encapsulated phenotype resulting from deletion of the sialic acid synthase 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 of 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 5A**, 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 (**Figures 5B** and **5C**, respectively), 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 6**, 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**).

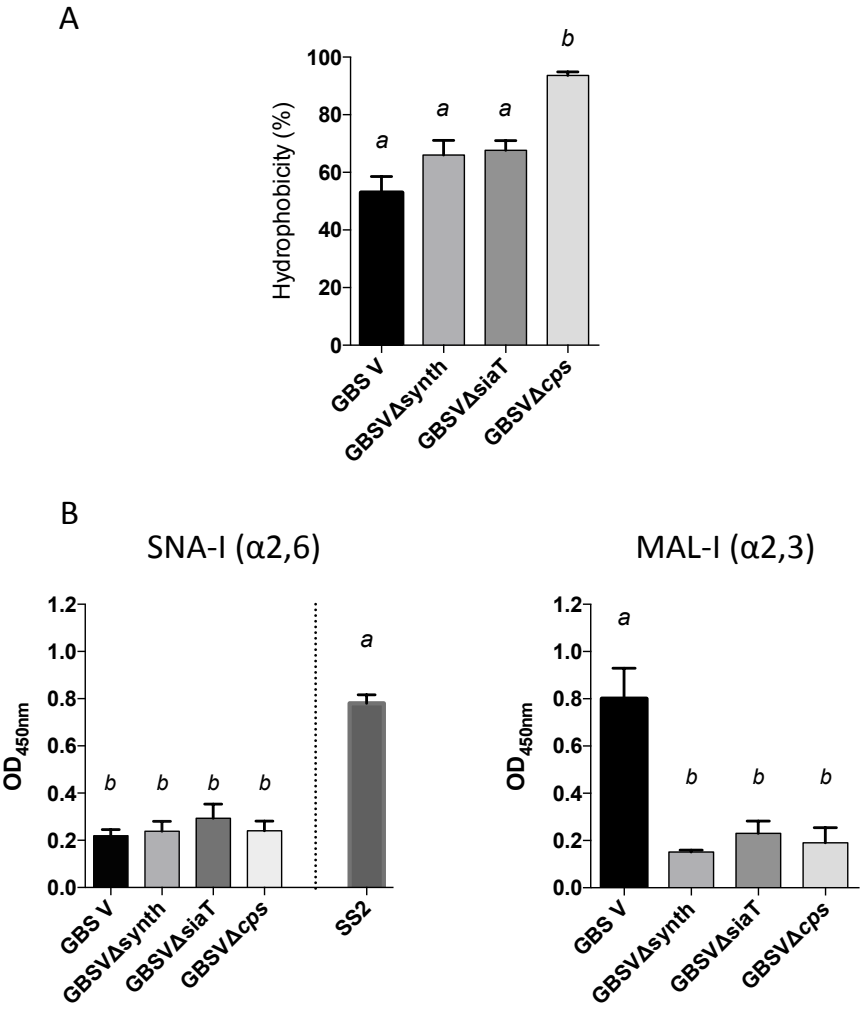


Figure 5. Capsular polysaccharide (CPS) expression levels and recognition of specific CPS sialic acid linkage in GBS type V isogenic mutants. (A) Hydrophobicity (%) of the wild-type GBS serotype V strain (GBS V), and the sialic acid synthesis GBSVΔsynth (Δ*neu5B*) and sialyltransferase GBSVΔsiaT (Δ*cps5K*) deficient mutants. The non-encapsulated strain (GBSVΔ*cps*) was used as control. (B-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 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), (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' ($P < 0.05$).

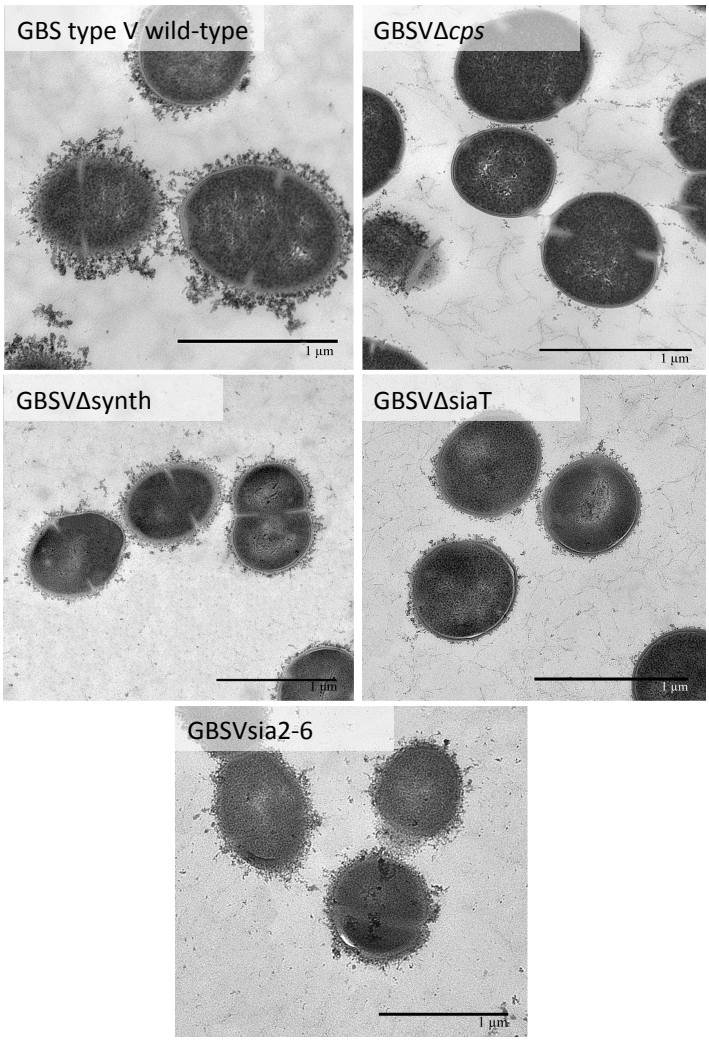


Figure 6. Transmission electron micrographs showing capsular polysaccharide (CPS) expression by GBS type V isogenic mutants. The CPS was labelled with polycationic ferritin. GBS type V wild-type strain was surrounded by a consistent CPS layer, whereas the GBSVΔsiaT (Δ*cps5K*), the GBSVΔsynth (Δ*neu5B*), 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.

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 ^1H spectrum of the GBSVΔsynth (Δ*neu5B*) mutant CPS (**Figure 7B**) represented *ca.* 0.2 equivalent compared to that of the wild-type GBS type V CPS (**Figure 7A**). These signals were totally absent from the spectrum of the GBSVΔsiaT (Δ*cps5K*) mutant CPS (**Figure 7C**), which in fact was essentially

identical to that of the chemically desialylated GBS type V polysaccharide (**Figure 7D**) (Calzas et al., 2013).

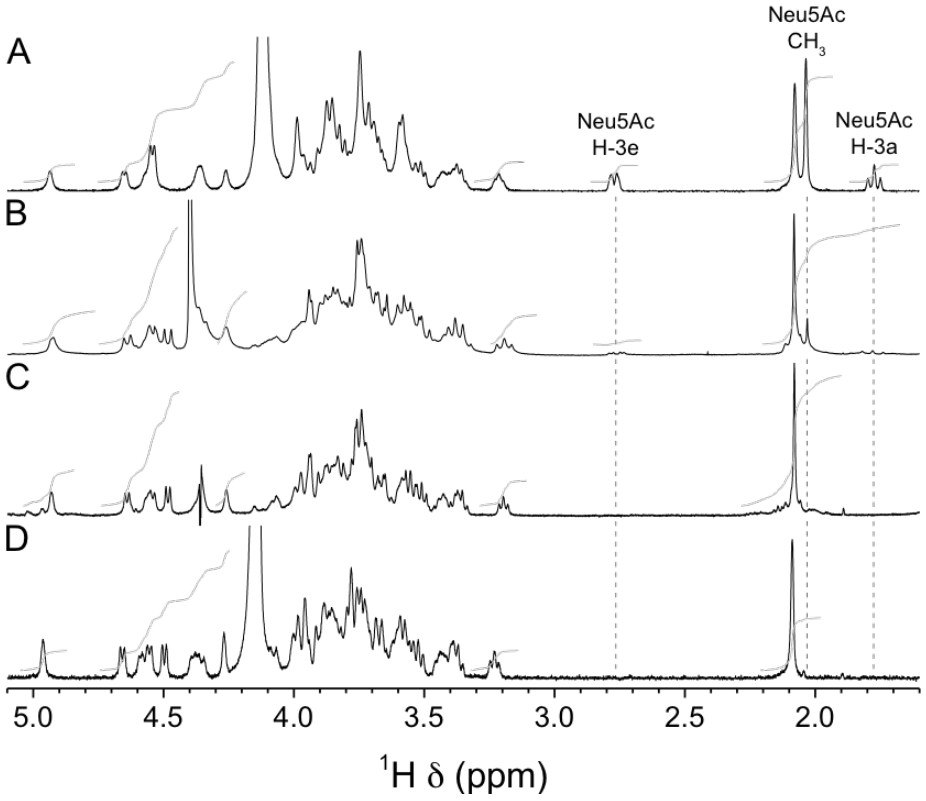


Figure 7. 1H NMR spectra of native GBS type V and sialic acid-deficient mutant capsular polysaccharides (CPSs). (A) Wild type, native CPS, D2O, 500 MHz, 30° pulse, 80°C. (B) GBSVΔsynth (Δ*neu5B*) mutant, native CPS, D2O, 300 MHz, 90° pulse, 60°C. (C) GBSVΔsiaT (Δ*cps5K*) mutant, native CPS, 33 mM phosphate pD 8.0 in D2O, 500 MHz, 90° pulse with presaturation, 67°C. (D) Wild type, chemically desialylated polysaccharide, D2O, 500 MHz, 30° pulse, 80°C.

Substitution of GBS α2-3 sialyltransferase by* S. suis *α2-6 sialyltransferase in GBS results in successfully modified sialic acid linkage

In order to study the specific role of sialic acid linkage in GBS CPS expression, 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 8A**, 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 8B and 8C**) 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 8B**). 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 8C**).

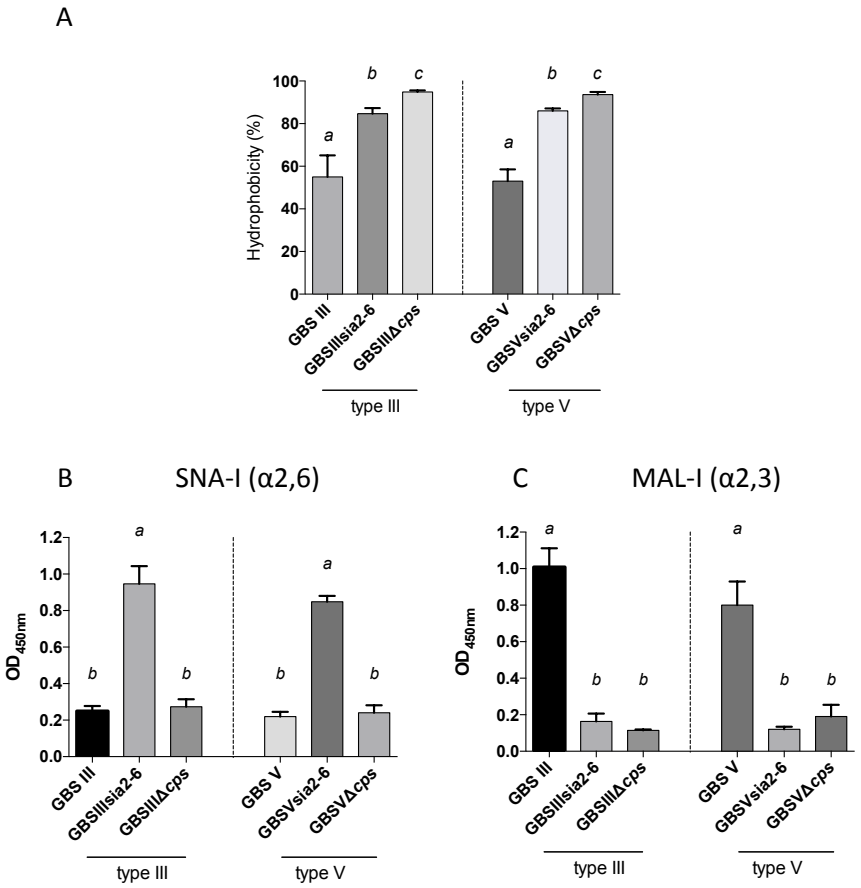


Figure 8. Capsular polysaccharide (CPS) expression levels and sialic acid linkage in GBS types III and type V mutants carrying exogenous α-2,6-sialyltransferase. (A) Hydrophobicity (%) of GBS type III and V wild-type strains and the mutants GBSIIIsia2,6 (Δ*cps3K/cps2N*) and GBSVsia2,6 (Δ*cps5K/cps2N*) 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' ($P < 0.05$).

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 9**) and GBSVsia2,6 (**Figure 6**), whereas a thicker CPS was observed for respective wild-type strains. In contrast, non-encapsulated control strains GBSIIIΔ*cps* (**Figure 9**) and GBSVΔ*cps* (**Figure 6**) 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**).

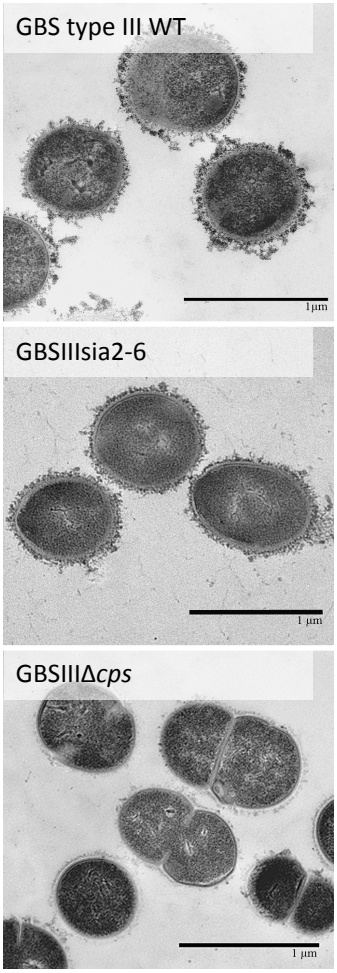


Figure 9. Transmission electron micrographs showing capsular polysaccharide (CPS) expression by GBS type III mutant carrying exogenous α-2,6-sialyltransferase. The CPS was labelled with polycationic ferritin. GBS type III wild-type strain was surrounded by a consistent CPS layer, whereas

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.

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 10**), consistent with sialic acid being 2,6- instead of 2,3-linked to the galactose residue (Machytka et al., 1994). 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**).

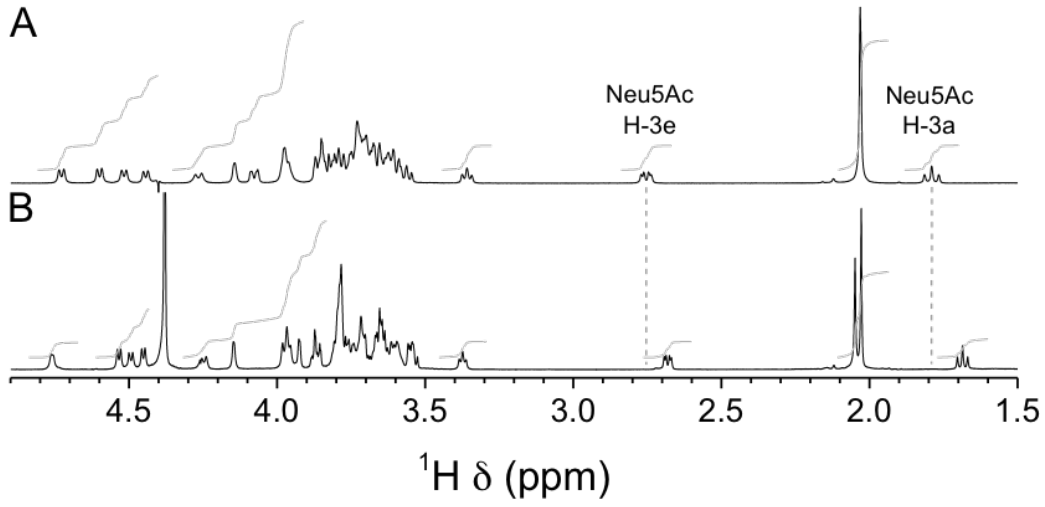
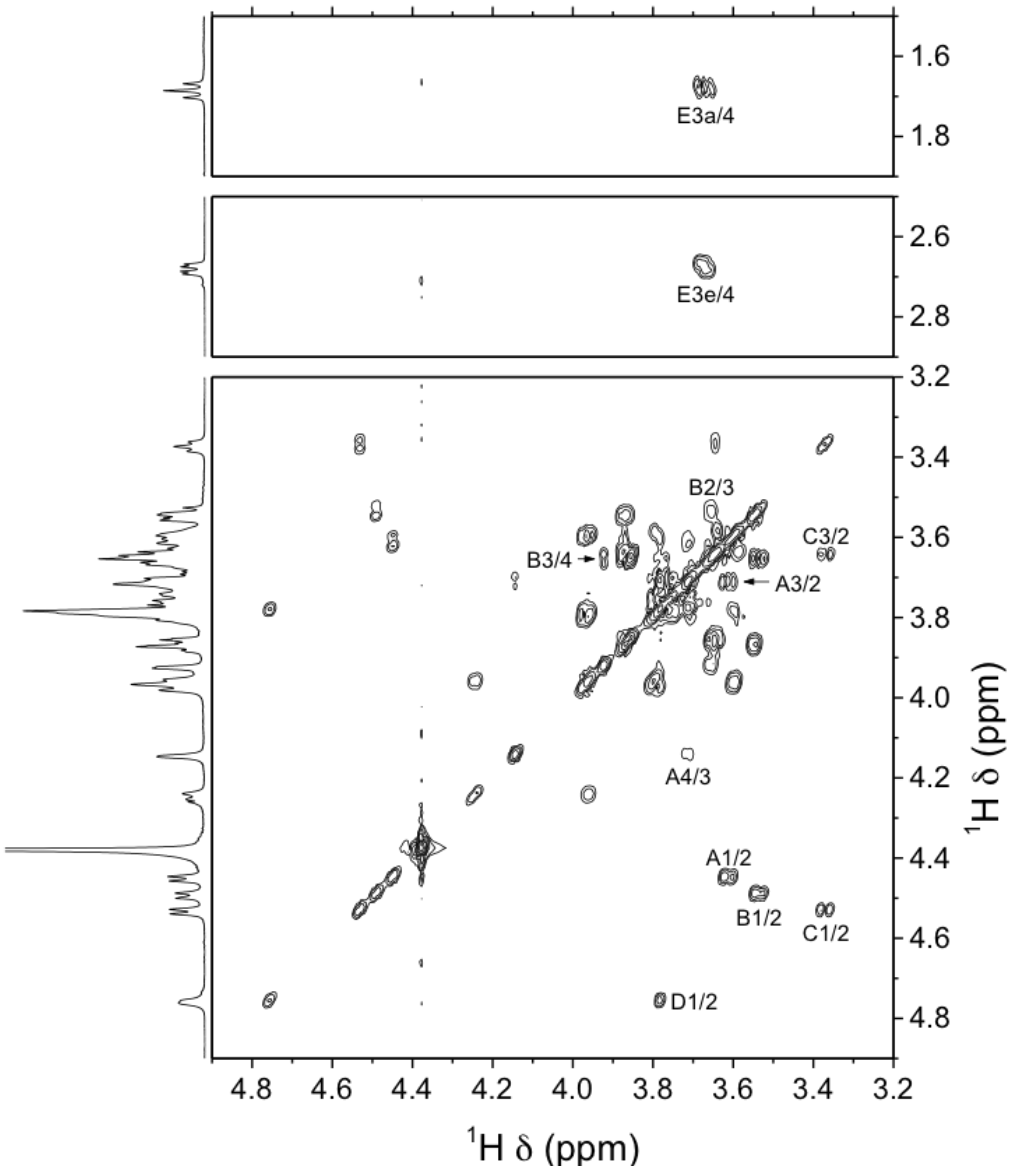
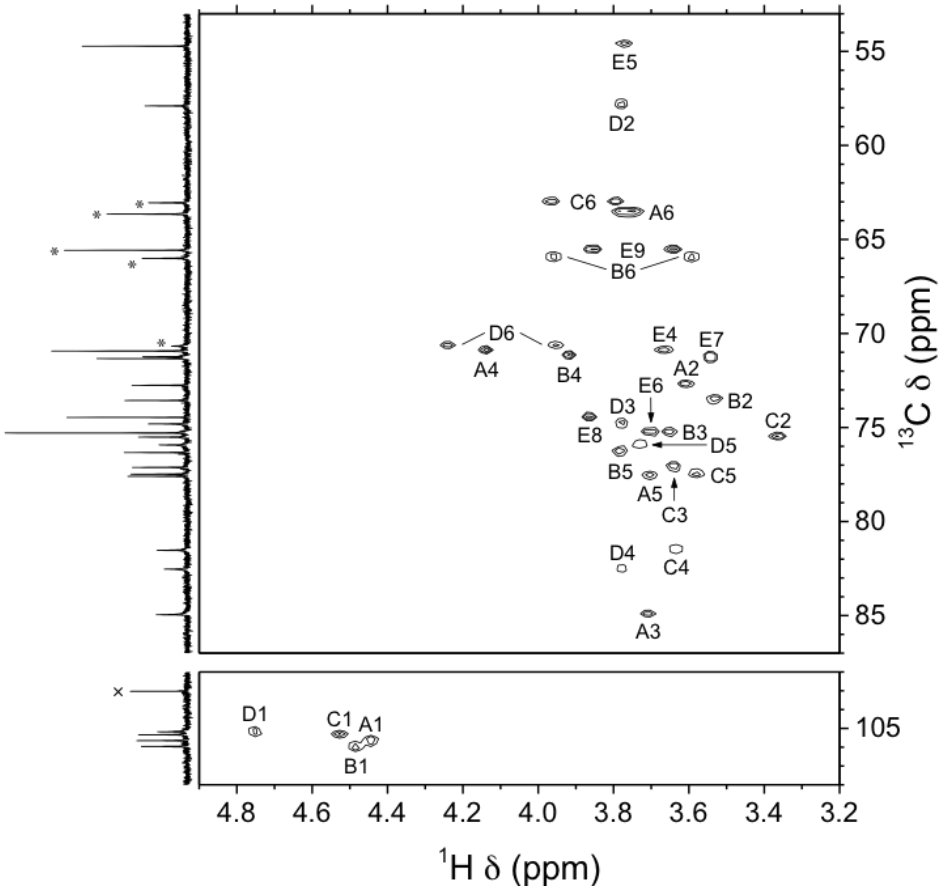


Figure 10. 1H NMR spectra of native GBS type III and mutant GBSIIIsia2,6 capsular polysaccharide (CPSs). (A) wild type GBS type III, D2O, 500 MHz, 90° pulse with presaturation, 62°C. (B) GBSIIIsia2,6 (Δ*cps3K/cps2N*) mutant, 33 mM phosphate pD 8.0 in D2O, 700 MHz, 90° pulse, 65°C.



Supplementary Figure S1. Portions of the 700-MHz 2D NMR COSY spectrum of GBSIIIsia2-6 mutant CPS in 33 mM phosphate pD 8.0 in D_2O at 65 °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. The t_2 dimension was processed by multiplication with an unshifted sine bell window function and Fourier transform, and the t_1 dimension was processed by Zhu-Bax linear prediction to 1024 points, multiplication with an unshifted sine bell window function, Fourier transform, and magnitude calculation. The f_1 trace corresponds to the 1D ^{1}H spectrum (see Fig. 11B).

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.



Supplementary Figure S2. Portions of the 176-MHz ge-2D NMR HSQC spectrum of GBSIIIsia2-6 mutant CPS in 33 mM phosphate pD 8.0 in D_2O at 65 °C. 2 × 200 increments of 512 complex data points were acquired in the echo–antiecho mode with a digital resolution of 9.8 Hz/point in the t_2 dimension and 57.3 Hz/point in the t_1 dimension. The t_2 dimension was processed by multiplication with a π/2 shifted sine bell window function, Fourier transform, and phase correction, and the t_1 dimension was processed by Zhu-Bax linear prediction to 400 points, multiplication with a π/2 shifted sine bell window function, zero filling, Fourier transform, and phase correction. Only positive contours are shown. The f_1 trace corresponds to the 1D ^{13}C spectrum. *, signals inverted on the DEPT spectrum; ×, signal absent on the DEPT spectrum.

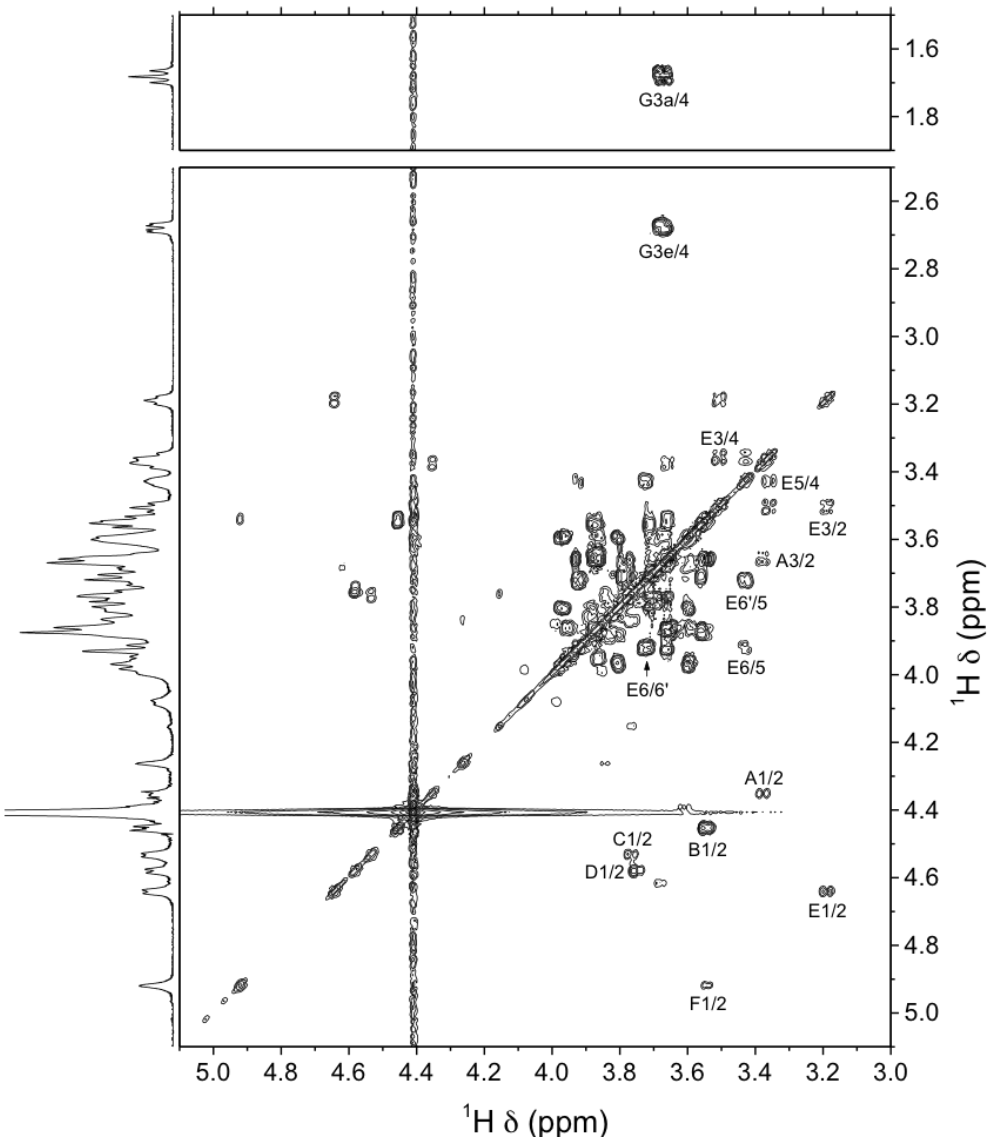
The DEPT spectrum (**Supplementary Figure S2** trace) confirmed a linkage at position 6 for two sugar residues. Carbons were assigned using the HSQC (**Supplementary Figure S2**) and HSQC–TOCSY (not shown) spectra, which also allowed identifying previously unassigned proton resonances. Full ^{1}H and ^{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.

Supplementary Table S2. ^{1}H and ^{13}C NMR chemical shifts of GBSIIIsia2-6 mutant CPS[a]

Residue		1		2	3		4	5	6		7	8	9		CH_3	CO
A	→3)-β-D-Gal-(1→	4.45	(7.7)[b]	3.62	3.72		4.15	3.71	3.79	3.76						
		105.65		72.76	84.96		70.93	77.60	63.65							
B	→6)-β-D-Gal-(1→	4.49	(8.8)	3.54	3.66		3.93	3.79	3.96	3.60						
		105.96		73.56	75.29		71.24	76.33	66.01							
C	→4)-β-D-Glc-(1→	4.53	(7.8)	3.38	3.65		3.64	3.59	3.97	3.80						
		105.34		75.51	77.12		81.53	77.48	63.05							
D	→3,6)-β-D-GlcNAc-(1→	4.76		3.78	3.78		3.78	3.74	4.24	3.96					2.05	
		105.18		57.90	74.81		82.53	75.93	70.67						25.07	177.45
E	α-D-Neu5Ac-(2→				2.68	1.69	3.67	3.78	3.70		3.55	3.87	3.86	3.65	2.03	
		175.97		103.03	42.82		71.34	54.72	75.29		70.93	74.46	65.58		24.77	177.64

[a] Chemical shifts (^{1}H, top; ^{13}C, bottom) at 65 °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 from the 1D, COSY, TOCSY, or HSQC spectrum. ^{13}C chemical shifts were obtained from the 1D spectrum.
[b] Coupling constants ($J_{H\text{-}1–H\text{-}2}$) in parentheses.

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 (Brisson et al., 1997), but also its linkage position exerts conformational control over the CPS backbone. The structure was also built in the program CASPER (Lundborg and Widmalm, 2011), and root-mean-square errors between experimental and calculated ^{1}H and ^{13}C chemical shifts were 0.03 and 0.25 ppm, respectively.



Supplemental Figure S3. Portions of the 700-MHz 2D NMR COSY spectrum of GBSVsia2-6 mutant CPS in 33 mM phosphate pD 8.0 in D_2O at 61 °C. 512 increments of 1 K complex data points were acquired in magnitude mode with a digital resolution of 4.2 Hz/point in the t_2 dimension and 8.3 Hz/point in the t_1 dimension. Processing was as for Supplemental Fig. S1. The f_1 trace corresponds to the 1D ^{1}H spectrum (see Fig. 12B).

Supplementary Table S3. ^{1}H and ^{13}C NMR chemical shift differences between GBSIIIsia2-6 mutant and wild-type GBS type III CPSs[a]

Residue	1	2	3		4	5	6		7	8	9	
A	0.01	0.02	0.00		−0.03	−0.01	−0.01	0.01				
	−0.20	0.01	−0.29		−0.09	−0.11	−0.15					
B	−0.14	−0.04	−0.45		−0.05	0.09	0.18	−0.15				
	1.16	1.40	−3.23		0.88	−1.45	2.32					
C	−0.02	0.01	−0.03		−0.04	−0.09	−0.04	−0.02				
	−0.03	0.16	0.07		0.37	0.02	0.26					
D	0.05	−0.04	0.05		−0.16	0.02	−0.06	−0.01				
	−0.72	−0.04	0.03		3.20	0.10	0.52					
E			−0.09	−0.14	−0.03	−0.09	0.03		−0.07	−0.03	−0.03	−0.02
	−0.66	0.18	0.40		0.17	0.27	−0.48		0.18	−0.14	0.23	

[a] Chemical shift differences (^{1}H, top; ^{13}C, bottom) in ppm. Data for wild-type GBS type III 14-repeating unit polysaccharide from Brisson *et al.* (1997) [25].

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 11**), 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**.

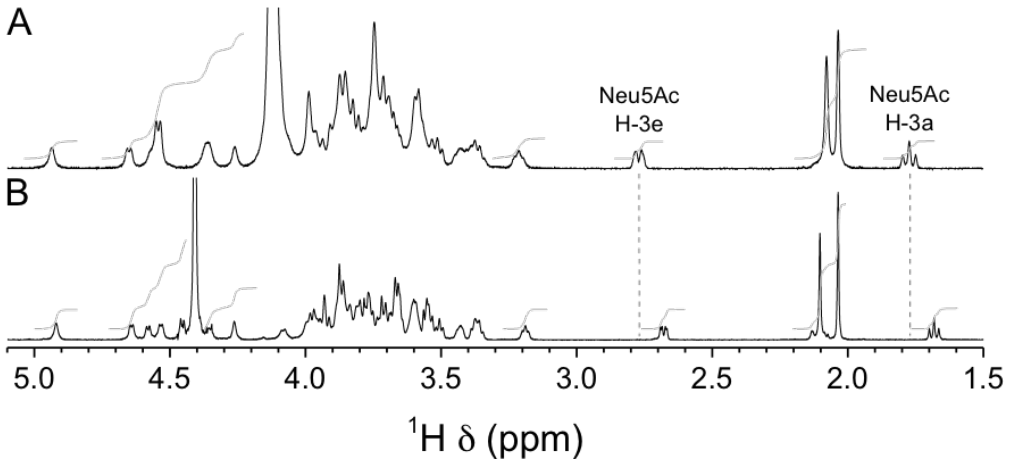
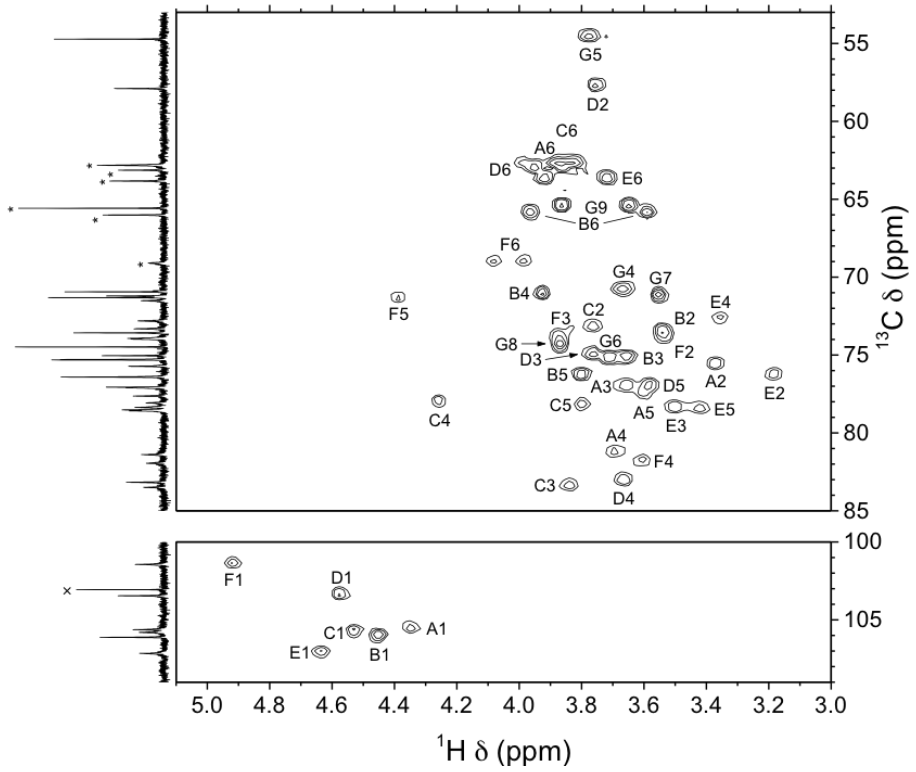


Figure 11. 1H NMR spectra of native GBS type V and mutant GBSVsia2,6 capsular polysaccharides (CPSs). (A) Wild type GBS type V, D2O, 500 MHz, 30° pulse, 80°C. (B) GBSVsia2,6 (Δ*cps5K/cps2N*) mutant, 33 mM phosphate pD 8.0 in D2O, 700 MHz, 30° pulse, 61°C.



Supplemental Figure S4. Portions of the 176-MHz ge-2D NMR HSQC spectrum of GBSVsia2-6 mutant CPS in 33 mM phosphate pD 8.0 in D_2O at 61 °C. 2 × 200 increments of 425 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 79.1 Hz/point in the t_1 dimension. Processing was as for Supplemental Fig. S2. Only

positive contours are shown. The f_1 trace corresponds to the 1D ^{13}C spectrum. *, signals inverted on the DEPT spectrum; ×, signal absent on the DEPT spectrum.

Supplementary Table S4. ^{1}H and ^{13}C NMR chemical shifts of GBSVsia2-6 mutant CPS[a]

Residue		1		2	3		4	5	6		7	8	9		CH_3	CO
A	→4)-β-D-Glc-(1→	4.35		3.38	3.66		3.70	3.61	3.99	3.85						
		105.63		75.72	77.11		81.39	77.63	62.82							
B	→6)-β-D-Gal-(1→	4.45	7.7	3.55	3.66		3.93	3.81	3.97	3.60						
		106.12		73.58	75.29		71.21	76.41	66.01							
C	→3,4)-β-D-Gal-(1→	4.53	7.1	3.77	3.85		4.26	3.81	3.88	3.84						
		105.81		73.31	83.49		78.08	78.36	62.82							
D	→4)-β-D-GlcNAc-(1→	4.58	7.6	3.76	3.77		3.67	3.59	3.96	3.87					2.10	
		103.45		57.89	75.04		83.17	77.06	63.13						25.53	176.67
E	β-D-Glc-(1→	4.64	7.4	3.19	3.51		3.36	3.43	3.93	3.73						
		107.15		76.41	78.52		72.81	78.59	63.83							
F	α -D-Glc-(1→	4.92		3.54	3.88		3.61	4.39	4.08	3.99						
		101.44		73.98	73.93		81.96	71.51	69.09							
G	α-D-Neu5Ac-(2→				2.68	1.68	3.67	3.78	3.71		3.56	3.88	3.87	3.65	2.04	
		175.98		103.06	42.84		70.94	54.72	75.31		71.32	74.48	65.78		24.86	177.66

[a] Chemical shifts (^{1}H, top; ^{13}C, bottom) at 61°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 from the 1D, COSY, TOCSY, or HSQC spectrum. ^{13}C chemical shifts were obtained from the 1D spectrum.
[a] Coupling constants ($J_{H\text{-}1\text{–}H\text{-}2}$) in parentheses.
[c] Tentative assignments.

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.

Only anomeric proton chemical shifts have been reported for the wild-type type V CPS (Wessels et al., 1991), so most chemical shift differences, and as a consequence conformational control, cannot be evaluated in this case. Again, the structure was built in the program CASPER, and root-mean-square errors between experimental and calculated ^{1}H and ^{13}C chemical shifts were 0.09 and 0.82 ppm, respectively.

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 biochemical characteristics and similarities in the *cps* loci, sialic acid exerts differential control of CPS expression by *S. suis* and GBS.

Compared to GBS, CPS structures and *cps* loci have been less studied in *S. suis*. To date, there is 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 (Chaffin et al., 2000). 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* (Smith et al., 1999; Lecours et al., 2012; Roy et al., 2015). 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, we developed an indirect three-step mutagenesis approach based on double cross-over homologous recombination to delete *S. suis cps2N* (Lakkitjaroen et al., 2014). 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* (Xayarath and Yother, 2007; Lakkitjaroen et al., 2014). In contrast, some of these mutations are known to be non-lethal for GBS type III, as it is the case for

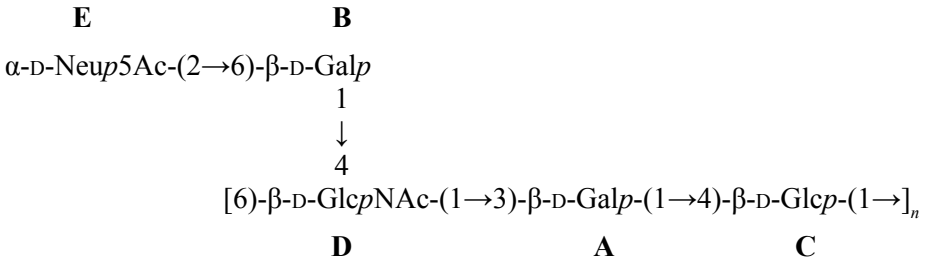
mutations in the sialyltransferase gene (*cpsK*) (Chaffin et al., 2005), suggesting differences in CPS synthesis between *S. suis* and GBS. The lethality of these mutations in *S. suis* and *S. pneumoniae* is hypothesized to be due to the sequestration of undecaprenyl-phosphate carrier CPS precursor 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* (Lakkitjaroen et al., 2014). Giving the fact that deletion of the *neuC* gene also inhibits CPS synthesis (Lecours et al., 2012), we used it as suppressor mutation in order to indirectly knockdown *cps2N*. Unfortunately, *S. suis* type 2 sialyltransferase gene deletion resulted in a non-encapsulated phenotype, confirming the critical role of sialic acid in the CPS expression by *S. suis*. 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 (Chaffin et al., 2005; Lecours et al., 2012) and GBS type V (this work) were 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 Chaffin *et al*. (Chaffin et al., 2005), 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 (Chaffin et al., 2005). 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.

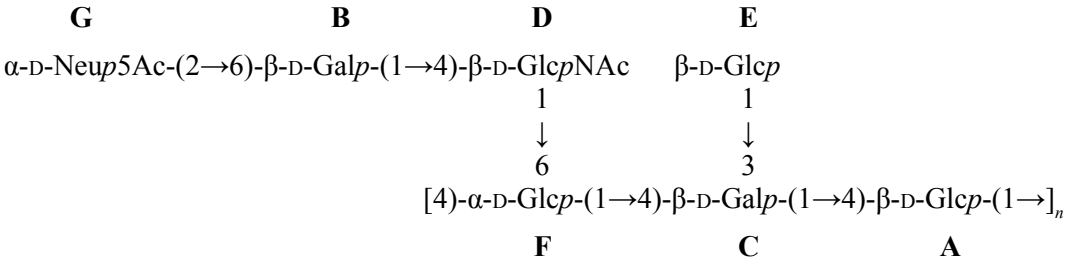
To evaluate how the specificity of the sialyltransferase and the resulting sialic acid linkage affect CPS expression, we performed for the first time inter-species sialyltransferase exchange between these two Gram-positive bacterial species. Since aforementioned differences between GBS types III and 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, suggesting that the *S. suis* sialyltransferase may recognize a common epitope/component (Okura et al., 2013). 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 (Chaffin et al., 2005). 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 (Chaffin et al., 2005).

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 *S. suis* enzymes. 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.



Scheme 1. Structure of GBSIIIsia2-6 mutant CPS with residue labels.



Scheme 2. Structure of GBSVsia2-6 mutant CPS with residue labels.

Conclusion

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. The modified GBS type III and V CPSs represent new tools to study CPS immunogenicity and host-pathogen interactions.

Experimental procedures

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.

Table 1. Bacterial strains and plasmids used in this study

Strain/Plasmid	General Characteristics	Source/ Reference
Escherichia coli		
TOP 10	F-*mrcA* Δ*(mrr-hsd*RMS-*mcr*BC*)ϕ80 lacZ*Δ*M5* Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
MC1061	*araD139 Δ*(*ara-leu*)*7697 Δlac*X74 *galU galK hsdR2*(rK-mK+) *mcrB1 rpsL*	[31]
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	[32]
SS2Δ*cps*	Non-encapsulated isogenic mutant strain derived from strain P1/7. In frame deletion of the *cps2F* gene	[12]
SS2ΔsiaT	Isogenic mutant strain derived from strain P1/7. Indirect in frame deletion of the *cps2N* gene (sialyltransferase)	This work
SS2compΔsiaT	Mutant SS2ΔsiaT complemented with pMX1*cps2N*	This work

SS2sia2-3	Isogenic mutant strain derived from strain P1/7. Indirect substitution of sialyltransferase *cps2N* gene by *cps3K* gene of GBS type III	This work
SS2Δsynth	Non-encapsulated isogenic mutant strain derived from strain P1/7. In frame deletion of the *neu2C* gene (sialic acid synthase)	[12]
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 *cps2N* gene by *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	[33]
SS14Δ*cps*	Non-encapsulated isogenic mutant strain derived from strain DAN13730. In frame deletion of the *cps14B* gene	[21]
SS14sia2-3	Isogenic mutant strain derived from strain DAN13730. Indirect substitution of sialyltransferase *cps14N* gene by *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 (sialic acid synthase)	[21]
SS14Δsynth/sia2-3	Isogenic mutant strain derived from strain DAN13730. In frame deletion of the *neu14C* gene and substitution of *cps14N* gene by *cps3K* gene of GBS type III	This work
Group B Streptococcus (GBS) type III		
GBSIII WT	COH1 wild-type (WT), well encapsulated type III strain isolated from an infant with sepsis and meningitis	[30]
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	[22]

GBSIIIΔsynth	Intermediate-encapsulated isogenic mutant strain derived from strain COH1. In frame deletion of the *neu3B* gene (sialic acid synthase)	[22]
Group B Streptococcus (GBS) type 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 sialyltransferase *cps5K* gene 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)	This work
GBSVΔsiaT	Isogenic mutant strain derived from strain CJB111. In frame deletion of the *cps5K* gene (sialyltransferase)	This work
GBSVΔ*cps*	Non-encapsulated isogenic mutant strain derived from strain CJB111. In frame deletion of the *cpsE* gene	[17]
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	[34]
pMX1	Replication functions of pSSU1, MCS pUC19 lacZ Sp^R, malX promoter of *S. suis,* derivative of pSET2	[34]
p4Δ*neuC*	pSET-4s carrying the construct for *neuC* allelic replacement (*S. suis* serotype 2 and 14)	This work
p4Δ*cps2N*	pSET-4s carrying the construct for *cps2N* indirect allelic replacement	This work
p4*neuC*	pSET-4s carrying intact *neuC* gene for *neuC* reintroduction (*S. suis* serotype 2 and 14)	This work
p4sia23_2	pSET-4s carrying construct for allelic replacement of *S. suis cps2N* gene by *cps3K* from GBS type III	This work

p4sia23_14	pSET-4s carrying construct for allelic replacement of *S. suis cps14N* gene by *cps3K* from GBS type III	This work
p4sia26_III	pSET-4s carrying construct for allelic replacement of GBS type III *cps3K* gene by *cps2N* from *S. suis* type 2	This work
p4sia26_V	pSET-4s carrying construct for allelic replacement of GBS type V *cps5K* gene by *cps2N* from *S. suis* type 2	This work
p4Δ*neu5B*	pSET-4s carrying construct for allelic deletion of GBS type V *neu5B* gene	This work
p4Δ*cps5K*	pSET-4s carrying construct for allelic deletion of GBS type V *cps5K* gene	This work
pMX1*cps2N*	pMX1 complementation vector carrying intact *cps2N* gene	This work

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: AF163833; 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).

Supplementary Table S1. Oligonucleotide primers used in this study

Oligonucleotide primers, sequence (5' – 3')[a]		Constructs or RTPCR reactions
cps2_ID1	CGTTGAATTTGTGGAACGGC	p4sia23_2
cps2_ID2	AGTTGCTCCCTGACATCTGG	p4sia23_2
cps2_ID3	GTAGCAGGTCTTGCCCCTTATC	p4sia23_2
cps2_ID4	GCAAGTGTGTAGCCGAAACTG	p4sia23_2
cps2_ID7	CCCAGTATCCCCCTTTATTTTC	p4sia23_2
cps2_ID8	CCAACAATTCGTGGTTCTCCTTCTACATAC	p4sia23_2
cps2_ID9	GGTGCAAGGATTAGCCGAAATGGCAGGTAG	p4sia23_2
cps2_ID10	CCATTACACGAGCGATGAAATC	p4sia23_2
cpsK_5	TTTATCAGCATTAGACGAGCG	p4sia23_2/14
cpsK_6	CCGGGCTGAACTTAAAGAACC	p4sia23_2/14
cpsK_11	GTATGTAGAAGGAGAACCACGAATTGTTGG	p4sia23_2/14
cpsK_12	CTACCTGCCATTTCGGCTAATCCTTGCACC	p4sia23_2/14
sia14_IDI	CGTTGAATTTGTGGAACGGC	p4sia23_14
sia14_ID2	AGTTGCTCCCTGACATCTGG	p4sia23_14
sia14_ID3	GTAGCAGGTCTTGCCCCTTATC	p4sia23_14
sia14_ID4	GCAAGTGTGTAGCCGAAACTG	p4sia23_14
sia14_ID7	CCCAGTATCCCCCTTTATTTTC	p4sia23_14
sia14_ID8	CCAACAATTCGTGGTTCTCCTTCTACATAC	p4sia23_14
sia14_ID9	GGTGCAAGGATTAGCCGAAATGGCAGGTAG	p4sia23_14
sia14_ID10	CCATTACACGAGCGATGAAATC	p4sia23_14
cpsJ_ID1	TGGTTCGACGGATAATTGTGC	p4sia26_III
cpsJ_ID2	CTGCTCCCGATAAGCAAACTC	p4sia26_III
cpsL_ID3	GTTGACACAGCCACTTGCAC	p4sia26_III
cpsL_ID4	CACCAGCAGCTAATAATGTCCC	p4sia26_III
cpsJ_ID7	GGGTTGTCAGAAGCTAGAAAC	p4sia26_III
cpsJ_ID8	TCTTGCCCTTCATCACCAACAATTCGTGGT	p4sia26_III
cpsL_ID9	GTTGTCTCAGGTCTCCATTCGTCCACTGCG	p4sia26_III
cpsL_ID10	CAGACACAGTGACAATGAACCG	p4sia26_III
cps2L_ID5	TCTTCTGCAAGTCACCTCACC	p4sia26_III
cps2L_ID6	ACGACCCAATCAGGCAAACC	p4sia26_III
cps2L_ID11	ACCACGAATTGTTGGTGATGAAGGGCAAGA	p4sia26_III
cps2L_ID12	CGCAGTGGACGAATGGAGACCTGAGACAAC	p4sia26_III
sia5_ID13	TGCAGTGGTGTATTTTAGCG	p4sia26_V
sia5_ID23	TCCGTCCTTATTCCCTGTTC	p4sia26_V
sia5_ID33	GCCGGTAGAGCTATTACCATC	p4sia26_V
sia5_ID43	TAACCAATTTACACCAGCAGC	p4sia26_V
sia5_ID73	AGGATTAGTGTGAAGGAGAAGG	p4sia26_V
5V_8	TTGCCCTTCATCATTGACACACAAAATTAT	p4sia26_V
5V_92	GATTCATGTCTAAAATGGGATAACACATTC	p4sia26_V
sia5_ID103	GTCTCCTCCCATTATTTGAGC	p4sia26_V
5V_11	ATAATTTTGTGTGTCAATGATGAAGGGCAA	p4sia26_V
5V_122	GAATGTGTTATCCCATTTTAGACATGAATC	p4sia26_V
cps2L3_ID1	GGGAGTTGGGAGTTACTATG	p4Δ*cps2N*
cps2L3_ID2	CTGACATCTGGAAAATGCC	p4Δ*cps2N*

cps2L3_ID3	AATGGCAGGTAGTATCCG	p4Δ*cps2N*
cps2L3_ID4	GACCGTTTTTCCCTGAATG	p4Δ*cps2N*
cps2L3_ID5	GGTAGATACTTTCATTGCGACC	p4Δ*cps2N*
cps2L3_ID6	CGTGAGGGGATAGAACAAGGAAATCAGGAT	p4Δ*cps2N*
cps2L3_ID7	ATCCTGATTTCCTTGTTCTATCCCCTCACG	p4Δ*cps2N*
cps2L3_ID8	GCAACAACAGATAGGAAGC	p4Δ*cps2N*
Neu14C_ID5	TCTCAGCTCGAAATGACTCGTC	p4*neuC*
Neu14C_ID8	AGGTCCCTGACTCCGTCAAC	p4*neuC*
Neu5B_ID1	TTATTGGTCTTCAGACGAGCGG	p4Δ*neu5B*
Neu5B_ID2	GCATCAACACCACAAGACACG	p4Δ*neu5B*
Neu5B_ID3	ATATTACGGTGAAGCGCCCAGG	p4Δ*neu5B*
Neu5B_ID4	GAGGAGGTTTCGACTGGTACAC	p4Δ*neu5B*
Neu5B_ID5	TTCGGTTCATTGTCACTGTGTC	p4Δ*neu5B*
Neu5B_ID6	GCGTGAATCACGAATGCAACCAATCTCTGC	p4Δ*neu5B*
Neu5B_ID7	GCAGAGATTGGTTGCATTCGTGATTCACGC	p4Δ*neu5B*
Neu5B_ID8	AGTACCGCTTTCATCTGCTCTC	p4Δ*neu5B*
cps5K_ID1	AGGATTAGTGTGAAGGAGAAGG	p4Δ*cps5K*
cps5K_ID2	TTTGTAACTGCTCCCGATAAGC	p4Δ*cps5K*
cps5K_ID3	TTTAGTGGGGCTACCTCATGAC	p4Δ*cps5K*
cps5K_ID4	AGCGCCATAGGCTGCATAATG	p4Δ*cps5K*
cps5K_ID5	GAAGATGCAATCGAGAGAATGG	p4Δ*cps5K*
cps5K_ID6	CGCGGTGGACGAATGCGTGATAGTGTCACA	p4Δ*cps5K*
cps5K_ID7	TGTGACACTATCACGCATTCGTCCACCGCG	p4Δ*cps5K*
cps5K_ID8	TCCAAGCGAATAACCGAAAC	p4Δ*cps5K*
cpsN_pstI_F	GCGCCTGCAGTATCGAAGCTGTACAGGG	pMX*cpsN*
cpsN_EcoRI_R	CGCGGAATTCCGAGACCTGAGACAACTATTG	pMX*cpsN*
M2000F	GATAGTTTGTCAGCCAGTGG	RTPCR (R1)
cpsA_R	GCCAATACTGCCACTCCTAC	RTPCR (R1)
M1000F	CTGATTGCACCGATTCGGAG	RTPCR (R3)
cpsA_R	GCCAATACTGCCACTCCTAC	RTPCR (R3)
M500F	CAATTCTGCCAATCCCTCTTG	RTPCR (R4)
cpsA_R	GCCAATACTGCCACTCCTAC	RTPCR (R4)
cpsA_F	GTCAAGCGATGGTGTTCAAC	RTPCR (R5)
cpsB_R	CACCGGCTTCAACATTTTG	RTPCR (R5)
cpsB_F	GCGGCACGTATTGCAAATAG	RTPCR (R6)
cpsD_R	GCACGAGCCATTAAGTGTTG	RTPCR (R6)
cpsD_F	GGCAGTAGCAGAAGTTTATCC	RTPCR (R7)
cpsF_R	GTAGCCATTCATGACCGTC	RTPCR (R7)
cpsF_F	GTTGGACGATTTGTGCCTG	RTPCR (R8)
cpsG_R	ACAGCCTGTGAAACTGTCAC	RTPCR (R8)
cpsF3_F	TTTTCATGGTCACGAGGTTG	RTPCR (R9)
cpsH_R	GTGTGATGTCCCATCGATAGC	RTPCR (R9)
cpsH_F	GCGAGAGAAGCCTCTTATTC	RTPCR (R10)
cpsJ_R	CATTTCCTAAGTCTCGCACC	RTPCR (R10)
cpsJ_F	GATAGTGATTTGTCGGGAGGG	RTPCR (R11)
cpsK_R	GCCATAATTACGGGCATCTG	RTPCR (R11)
cpsK_F	CGCCAAGGGTGACTACTTAG	RTPCR (R12)
cpsN_R	CGAGCGACAGATCATTGACC	RTPCR (R12)
cpsN_F	GCTCGTACAATTTGACGAGG	RTPCR (R13 and R14)
cpsO_R	CAGGCAAACCAAATAGGAGC	RTPCR (R13)
neu1_R	ATCGGTCGTTCCATTTTCCTG	RTPCR (R14 and R15)
cpsO_F	CGCTCCTATTTGGTTTGCC	RTPCR (R15)
neu2_F	GGAAAAAATTGGTCGTCAAGC	RTPCR (R16)
neu2_R	TGGCAACTGGTAGCATCTC	RTPCR (R16)
neu3_F	CCTTGACAGAGCAACTCAC	RTPCR (R17)
neu3_R	ACCAAGGAAGGACACCATC	RTPCR (R17)
neu4_F	GGCCTCAACCATGAAAGAG	RTPCR (R18)

neu4_R	GTTGTAAAATCTGTCGCCAAG	RTPCR (R18)
neu5_F	TTTTCCATACCATTCGAGCTG	RTPCR (R19)
neu5_R	TGCTCTGCTTTGGAAACAAG	RTPCR (R19)
neu6_F	ATCGGTGGGATGACAGCTTC	RTPCR (R21, R22, R23, R24 and R25)
neu6_R	TCCTTTTCACGACCTGACTTG	RTPCR (R21)
neu6R_R	TGGTCAAACTTGTCAAAATC	RTPCR (R22)
neu8R_R	TACTTGTTCGGAAGTTAGAGCC	RTPCR (R23)

[a] Oligonucleotide primers were from IDT (Coralville, IA); restriction sites are underlined.

RT-PCR analysis of 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.

*Indirect deletion of sialyltransferase (*cps2N*) in* S. suis

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*. First, precise in-frame deletion in the *neu2C* gene (sialic acid synthase) was achieved using splicing-by-overlap-extension PCR (Warrens et al., 1997) as previously described, to generate the SS2Δsynth (Δ*neu2C*) mutant (Lecours et al., 2012; Roy et al., 2015).

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 μF), 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 genes was confirmed by PCR and sequence analysis. 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* (Lakkitjaroen et al., 2014).

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 (Warrens et al., 1997). 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 (Roy et al., 2016).

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 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 (Roy et al., 2016). Deletion/insertion of targeted genes was confirmed by PCR and sequence analysis.

Indirect substitution of sialyltransferase (*cpsN*) in* S. suis *serotype 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 (sialic acid synthases) 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* (Lakkitjaroen et al., 2014).

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 (Roy et al., 2016).

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.

Construction of complemented Δ*cps2N *mutant

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* (Takamatsu et al., 2001a). Complementation of the SS2ΔsiaT (Δ*cps2N*) 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.

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 (Warrens et al., 1997). 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* (Roy et al., 2016). Deletions of the *cps5K* and *neu5B* genes in GBSVΔsiaT and GBSVΔsynth, respectively, were confirmed by PCR and sequence analysis.

Exogenous sialyltransferase exchange in GBS type 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.

Hydrophobicity test

In order to investigate 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 (Bonifait et al., 2010). 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.

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 (Shibuya et al., 1987; Geisler and Jarvis, 2011). The test was based on a previously described technique and adapted for whole bacteria (Gornik and Lauc, 2007). A 10-ml overnight culture in THB inoculated with the appropriate strains was harvested by centrifugation and resuspended in 10 ml of PBS. The suspension was then diluted 10X in PBS (10^7 CFU/ml), and 100 µl 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.

Transmission electron microscopy

TEM was carried out to confirm CPS expression at the bacterial surface of different mutant strains as previously described (Roy et al., 2015). 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).

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 (Calzas et al., 2013). 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.

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%. ^{1}H 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 Wishart *et al.* (Wishart et al., 1995). A Chemagnetics (Fort Collins, CO) CMX Infinity 300 spectrometer was used for 7.05-T experiments with a 5-mm dual $^{13}C/^{1}H$ Nalorac probe (Martinez, CA) at 60°C. The one-dimensional (1D) ^{1}H 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 ^{1}H, ^{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 ^{1}H 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 ^{1}H spectra were acquired with 90° or 30° pulses with or without solvent presaturation. The *z*-restored spin-echo was used to acquire 1D ^{1}H-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 (Zhu, 1992).

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 Calzas *et al*. (Calzas et al., 2013). 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).

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.

Acknowledgments

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) PhD award and of a scholarship from the Swine and Poultry Infectious Disease Research Center.

Author contributions

MS, DT and DR designed research studies; DR and AD performed research experiments; GGD contributed to CPS purification and analysis. DR, MRVC, MS, MG analyzed the data; DR wrote the first draft of the manuscript. All authors reviewed and approved the manuscript.

Competing financial interests

The authors declare no conflict of interest.

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ARTICLE V

Recruitment of factor H to the *Streptococcus suis* cell surface is multifactorial

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Publié dans: Pathogens. **2016.** Jul 7;5(3). doi: 10.3390/pathogens5030047.

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 et à la réalisation des expériences, à l'analyse des résultats et à l'écriture de l'article.

Abstract

Streptococcus suis is an important bacterial swine pathogen and a zoonotic agent. Recently, two surface proteins of *S. suis*, Fhb and Fhbp, have been described for their capacity to bind factor H—a soluble complement regulatory protein that protects host cells from complement-mediated damages. Results obtained in this study showed an important role of host factor H in the adhesion of *S. suis* to epithelial and endothelial cells. Both Fhb and Fhbp play, to a certain extent, a role in such increased factor H-dependent adhesion. The capsular polysaccharide (CPS) of *S. suis*, independently of the presence of its sialic acid moiety, was also shown to be involved in the recruitment of factor H. However, a triple mutant lacking Fhb, Fhbp and CPS was still able to recruit factor H resulting in the degradation of C3b in the presence of factor I. Similarly to the wild-type strain, in the presence of complement factors, the double mutant lacking Fhb and Fhbp was phagocytosed by human macrophages and killed by pig blood. In conclusion, this study suggests that recruitment of factor H to the *S. suis* cell surface is multifactorial and redundant.

Introduction

Infections caused by *Streptococcus suis* represent an economic problem for the swine industry, being one the most important bacterial pathogen for weaned piglets [1]. It usually causes septicemia with sudden death, meningitis, arthritis, endocarditis and other infections [1]. Moreover, *S. suis* is a zoonotic agent causing meningitis, septicemia and toxic shock-like syndrome in humans [2]. In Western countries, human *S. suis* infections are mainly associated with the swine industry whereas in some Asian countries, the general population is at risk and *S. suis* is one of the main causes of adult meningitis [3]. Serotype 2 is the most virulent and the most commonly isolated serotype in swine and humans [4]. The pathogenesis of the *S. suis* infection is only partially known; it has been proposed that, in swine, bacteria first colonize epithelial cells of the respiratory tract, and then reach the bloodstream where they can survive and multiply [5]. The capsular polysaccharide (CPS), rich in sialic acid, plays an important antiphagocytic role [5]. In addition, *S. suis* possesses different surface proteins implicated in host colonization and resistance to host immune response. While several of these surface

proteins have already been characterized, many remain poorly studied and their physiological and pathological roles uncharacterized [5].

Factor H is a soluble complement regulatory protein that protects host cells from complement-mediated damages [6]. This plasma glycoprotein is the key fluid phase regulator of the alternative complement pathway and acts as a cofactor in the factor I-mediated proteolysis of C3b. Proteolytic cleavage of C3b results in the formation of the inactive iC3b fragment, which remains covalently linked to the surface [7]. It also competes with factor B for binding to C3b, therefore interfering with the formation of the C3bBb complex [7]. However, many bacterial pathogens have the ability to bind factor H to their cell surface in order to avoid complement attack and opsonophagocytosis. In several bacterial pathogens, surface-exposed proteins and/or sialic acid-rich polysaccharide components have been shown to be able to bind factor H [8]. In addition to its complement regulatory function—which may modulate opsonophagocytosis— factor H has been shown to contribute to bacterial adherence to different host cells [9].

It is known that *S. suis* is able to bind factor H as a cofactor in order to degrade C3b in the presence of complement factor I [10]. Two different surface proteins of *S. suis*, Fhb and Fhbp, have so far been described for their capacity to bind factor H [10,11]. Recombinant Fhb and Fhbp proteins have a molecular weight of 130 kDa, and 95 kDa, respectively [10,11]. Although both proteins share low protein identity (33%), they possess a LPXTG sequence followed by hydrophobic domains recognized by the sortase A anchoring enzyme [10,11]. So far, only Fhb was characterized for its role in the pathogenesis of *S. suis* in a piglet model [11]. However, *fhbp* expression was shown to be upregulated in brain and lungs during experimental infection of pigs with *S. suis* [12].

In order to evaluate the individual or combined contribution of Fhb, Fhbp as well as the CPS in the ability of *S. suis* to bind factor H, we constructed single isogenic as well multiple knock-out mutants of *S. suis* serotype 2 deficient for the above surface constituents. Our results show that binding of factor H to the *S. suis* surface increases adhesion to both epithelial and endothelial cells. However, recruitment of factor H to the bacterial surface is multifactorial.

Materials and Methods

Bacterial strains and culture conditions

The virulent serotype 2 strain P1/7 was used as the wild-type strain for in-frame allelic deletion mutagenesis. *Streptococcus mutans* strain 25175 (ATCC, Manassas, VA, USA) was used as a negative control for factor H deposition studies. Bacterial strains and plasmids used in this study are listed in Table I. *Streptococcus* strains were grown in Todd-Hewitt broth (THB) or agar (THA) (Becton-Dicksinson, Sparks, MD, USA) at 37 ºC. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or agar (Becton-Dickinson) at 37 ºC. When needed, antibiotics (Sigma-Aldrich Canada Co., 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 (Km) and Sp at 50 μg/mL; and ampicillin at 100 μg/mL.

Table I. Bacterial strains and plasmids used in this study

Strains/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* Δ*lac*X74 *galU galK hsdR2*(rK-mK+) *mcrB1 rpsL*	[38]
Streptococcus suis		
P1/7	Wild-type strain, highly encapsulated serotype 2 strain isolated from a clinical swine case of infection in the United Kingdom	[39]
Δ*cps2F*	Non-encapsulated isogenic mutant strain derived from strain P1/7. Deletion of the *cps2F* gene	[13]
Δ*fhb*	Fhb expression-deficient strain derived from strain P1/7. Deletion of the *fhb* gene (SSU0253)	This work
Δ*fhbp*	Fhbp expression-deficient strain derived from strain P1/7. Deletion of the *fhbp* gene (SSU0186)	This work
Δ*fhb*/Δ*fhbp*	Fhb and Fhbp expression-deficient strain derived from strain P1/7. Deletion of the *fhb* and *fhbp* genes	This work
Δ*fhb*/Δ*fhbp*/Δ*cps2F*	Non-encapsulated Δ*fhb*/Δ*fhbp* mutant derived from strain Δ*fhb*/Δ*fhbp*. Deletion of the *cps2F* gene	This work
comp Δ*fhbp*	Mutant Δ*fhbp* complemented with pMXfhbp complementation vector	This work
Streptococcus mutans 25175	Wild-type strain, isolated from a carious dentine case	ATCC 25175
Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Invitrogen
pSET-4s	Thermosensitive vector for allelic replacement. Replication functions of pG+host3, MCS *oriR* pUC19 *lacZ* Sp^R	[40]
pMX1	Replication functions of pSSU1, MCS pUC19 lacZ Sp^R, malX promoter of *S. suis,* derivative of pSET2	[40,41]
p4Δ*fhb*	pSET-4s carrying the construct for *fhb* allelic replacement	This work

p4Δ*fhbp*	pSET-4s carrying the construct for *fhbp* allelic replacement	This work
p4Δ*cps2F*	pSET-4s carrying the construct for *cps2F* allelic replacement	This work
pMXfhbp	pMX1 complementation vector carrying intact *fhbp* gene	This work

Cell lines and cell culture

The human lung epithelial cell line A549 (ATCC CCL-185) was used and cultured until confluent as previously described [19]. The human brain microvascular endothelial cell line (hBMEC, gift from Dr. K. S. Kim, Johns Hopkins University School of Medicine, MD, USA) was used and cultured until confluent as previously described [42]. THP-1 human monocytic cell line (ATCC TIB-202) was used and cultured as previously described [43].

DNA manipulations

S. suis genomic DNA was purified using InstaGene Matrix solution (BioRad Laboratories, Hercules, CA, USA). Oligonucleotide primers used in this study are listed in Table II. Transformations of *E. coli* were performed following the manufacturers' recommendations (Invitrogen, Burlington, ON, Canada). Extraction and purification of recombinant plasmids were performed using QIAprep Spin Miniprep kit (Qiagen Valencia, CA, USA). Restriction enzymes and DNA-modifying enzymes were purchased from Fisher Scientific (Ottawa, ON, Canada) and used according to the manufacturers' recommendations. PCR reactions were carried out with an iProof high-fidelity DNA polymerase (BioRad Laboratories) or with Taq DNA polymerase (Qiagen). Oligonucleotide primers were ordered from Integrated DNA Technologies (IDT, Coralville, IA, USA). Amplification products were purified using 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, USA).

Table II: Oligonucleotide primers used in this study.

Oligonucleotide primers, sequence (5' – 3')[a]		Constructs
fhbp-ID1	ACTGACAACATGACCGACCTCC	p4Δ*fhbp*
fhbp -ID2	TGTTGAAGTCTCTGTCGTCGC	p4Δ*fhbp*
fhbp -ID3	AAGTCATAAGGGCGCACCTTC	p4Δ*fhbp*
fhbp -ID4	TGTAGCCAGCGATAAGGCTCTG	p4Δ*fhbp*
fhbp -ID5	AACAGCCAGGCTTATGGAAGG	p4Δ*fhbp*
fhbp -ID6	TATAGCTGTAGCGACACGAATACTATATCT	p4Δ*fhbp*
fhbp -ID7	AGATATAGTATTCGTGTCGCTACAGCTATA	p4Δ*fhbp*
fhbp -ID8	TGTCAAGCCAATCCATGTCTGG	p4Δ*fhbp*
fhb -ID1	TCGGTGCTATCTTGCGTAGTC	p4Δ*fhb*
fhb -ID2	CATCTGGTTCTAGCGATTCTGC	p4Δ*fhb*
fhb -ID3	TGATGCCAAAAGCAGAGGCAC	p4Δ*fhb*
fhb -ID4	TGGAACTTTCGAGGTCGGTG	p4Δ*fhb*
fhb -ID5_EcoRI[b]	GGCGCGAATTCCAAAGTTCTTGCCAGATGCCAC	p4Δ*fhb*
fhb -ID6	CCAGCCTATTGCGCTCCCTAATACGACTGT	p4Δ*fhb*
fhb -ID7	ACAGTCGTATTAGGGAGCGCAATAGGCTGG	p4Δ*fhb*
fhb -ID8_PstI[b]	GGCGCCTGCAGAAATTTCCGCCCCTGACACAC	p4Δ*fhb*
pFHBP_F_PsI[b]	GCGCCTGCAGCACATCCGACCACCTGAATATC	pMXfhbp
pFHBP_R_PstI[b]	GGCGCCTGCAGGTTCTAAAAAGAGGCTGGGCG	pMXfhbp
cps -ID1	CCAGCAAAGTATGGTGGTTTCG	p4Δ*cps2F*
cps -ID2	GCGCACCAACTTCTCTTAATGC	p4Δ*cps2F*
cps -ID3	CTTAGTCACTCCGAACTCACCG	p4Δ*cps2F*
cps -ID4	CCACGCCAGATTCAATGAGC	p4Δ*cps2F*
cps -ID5	AGACGGTCATGAATGGCTACG	p4Δ*cps2F*
cps -ID6	GAGGGAGGTGTAGACTTCTGCTCCAGCATG	p4Δ*cps2F*
cps -ID7	CATGCTGGAGCAGAAGTCTACACCTCCCTC	p4Δ*cps2F*
cps -ID8	CATCAGAATGATGCCAAACAGG	p4Δ*cps2F*

[a] Oligonucleotide primers were from IDT.
[b] Restriction sites are underlined.

Construction of allelic deletion mutants

Fhb protein and CPS deletion mutants (*fhb* and *cps2F*) have been previously obtained and characterized [11,13]. Fhbp, double Fhb/Fhbp and triple Fhb/Fhbp/CPS deletion mutants were obtained for the first time in the present study. Precise in-frame deletions were achieved using splicing-by-overlap-extension PCR [44]. Overlapping PCR-products generated by PCR were cloned into plasmid pCR2.1 (Invitrogen), extracted using EcoRI, and cloned into the thermosensitive *E. coli-S. suis* shuttle vector pSET4s previously digested with EcoRI, giving rise to the p4Δ*fhb*, p4Δ*fhbp* and p4Δ*cps2F* mutation vectors. Final constructions of 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 uF) and cells were plated on THA supplemented with Sp (THA+SP) and incubated for 3 days at 28 ºC. Several Sp-resistant colonies were then subcultured again on THA+SP for 3 days at 28 ºC. The candidates were next cultured on THA+SP and incubated at 37 ºC for two successive passages and then screened for first crossing-over event. Lost of vector was induced by incubation of candidates at 28 ºC. Temperature and Sp-resistant clones were successively cultured on THA and THA+SP to obtain Sp-sensitive candidates. Deletion of the genes was confirmed by PCR and sequence analysis.

The lack of expression of Fhbp in the Δ*fhbp* mutant was evaluated by immunoblot. Briefly, bacteria from 10 mL of overnight cultures of the wild-type, mutant and complemented strains under investigation were harvested by centrifugation and resuspended in PBS at OD_{600} = 0.600. Ten µL of bacteria were mixed with 10 µL of denaturing electrophoresis buffer and boiled for 10 minutes. Samples were then electrophoresed on sodium dodecyl sulfate-polyacrylamide (SDS) gels. Western-blotting was carried out as previously described by Burnette [45], using antisera from immunized rabbit with recombinant Fhbp protein, which was cloned, expressed and purified as previously described [10].

The presence of the CPS in Δ*fhb*, Δ*fhbp* and Δ*cps2F* mutants were tested by the coagglutination and dot-ELISA tests using anti-serotype 2 polyclonal and monoclonal

antibodies, respectively [46,47]. In addition, surface hydrophobicity was tested as previously described [34].

Construction of complemented *Δfhbp* mutant

Complemented Δ*fhb* and Δ*cps2F* mutants had previously been done [11,13]. Since the Fhbp mutant was obtained for the first time in this study, a complemented strain was also produced. Intact *fhbp* gene was amplified from genomic DNA of the wild-type strain with primers containing specific restriction sites (Table II). PCR products and pMX1 vectors were then digested with the appropriate restriction enzyme before ligation. Final constructions were cloned into *E. coli* MC1061. 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 [40]. Complementation of Δ*fhbp* mutant was achieved by transformation with pMXfhbp by electroporation under the same aforementioned conditions. Presence of the plasmid within the mutant was confirmed by PCR. Expression of the Fhbp was studied as described above.

Adhesion and invasion assays

The A549 and hBMEC cells were used to evaluate the adhesion properties of the different mutants and wild-type strains in presence or absence of factor H. Bacteria were grown to mid-logarithmic phase, harvested by centrifugation, washed three times and resuspended in PBS at OD_{600} = 0.600. One-hundred µL of bacterial suspension (equivalent to 1 x 10^7 CFU) were preincubated with 2 µg of human factor H (Quidel, San Diago, CA, USA) for 20 minutes at 37 °C. Four-hundred µL of RPMI were then added to bacteria without washing out unbound factor H, as previously described for other streptococci [19]. Cells were infected with *S. suis* strains (1 x 10^7 CFU/well, multiplicity of infection [MOI]: 50) and incubated at 37 °C in 5% CO_2 for 1 hour. An adhesion assay—which in fact quantifies total intracellular bacteria and surface-adherent bacteria—was performed as previously described [48]. Cell monolayers were washed five times with PBS and lysed with sterile cold water. Viable bacteria were determined by plating samples onto THA using an Autoplate® 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA). The invasion assay was performed using the antibiotic protection assay as previously described [48]. Briefly, after the initial incubation time, cell

monolayers were washed twice with PBS and incubated for an additional 1 h with medium containing 5 µg/mL penicillin G (Sigma) and 100 µg/mL of gentamicin (Gibco, Burlington, ON, Canada) in order to kill extracellular bacteria. Cell monolayers were then washed three times with PBS and lysed with sterile cold water. Viable intracellular bacteria were determined as described above. Optimal incubation time and MOI for both adhesion and invasion tests were chosen based on published studies [42,49] and preliminary tests done with the wild-type strain. Adhesion and invasion tests were done in duplicates and repeated at least four times in independent experiments.

Evaluation of factor H deposition to *S. suis* strains

Cell-based enzyme-linked immunosorbent assay (ELISA) was used to evaluate the factor H deposition at the bacterial surface of different mutants obtained in this study, as previously described with some modifications [10]. Briefly, washed harvested bacteria were adjusted to an OD_{600} of 0.2 in 50 mM carbonate buffer (pH 9.6). One-hundred µL/well were added to flat-bottom 96-well microplates (Nunc-immuno® Polysorp; Nalge Nunc International, Rochester, NY, USA) and incubated for 2 hours at room temperature. Bacterial suspension was removed and bound bacteria were fixed with glutaraldehyde (0.05%) for 45 minutes. Wells were then washed three times with PBS-Tween 0.05% (PBS-T) and blocked with PBS-T supplemented with 2% fat-free milk for 1 hour. After washing, fixed bacteria were then incubated in presence of 100 µL of human factor H (10 µg/mL in PBS) for 2 hours at 37 °C. Plates were then washed three times to remove unbound factor H. The deposition of factor H at the bacterial surface was detected by goat antisera against human factor H (Quidel) and HRP-conjugated donkey anti-goat IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA). The OD_{450} was recorded with a microplate reader after adding horseradish peroxydase substrate. Each assay was repeated in duplicates in four independent experiments.

Factor H recruitment by the capsular polysaccharide

In order to investigate the potential role of the CPS of *S. suis* serotype 2 as well as its sialic acid component in factor H recruitment, an ELISA was performed as previously described [50], with some modifications. Briefly, 100 µL of native or desialylated CPS (0.1 or 1 µg/mL

in carbonate buffer), highly purified as previously described [28], was used to coat wells of Polysorp flat-bottom 96-well microplate and incubated at 37 °C for 2 hours. Unbound CPS was washed with PBS-T three times and wells were blocked as described above. After washing, purified factor H (10 µg/mL, chosen based on preliminary dose-response studies; not shown) was added to the wells and incubated for 2 hours at room temperature. Correct binding of purified CPS to the wells was verified as described [50]. Deposition of factor H was detected as described above.

C3b cleavage assay

The functional activity of factor H bound to *S. suis* was assayed using a factor I–cofactor assay as described by Vaillancourt *et al*. [10]. Briefly, *S. suis* cells were incubated with human factor H (0.7 µg/mL) for 2 h at 37 °C. Bacteria were then washed three times in PBS and suspended in the same buffer, and human C3b (4.5 mg/mL; Calbiochem EMD chemicals, Billerica, MA, USA) as well as human factor I (2.5 mg/ mL; Quidel) were added. Following incubation at 37 °C for 2 h, denaturing electrophoresis buffer was added and the mixture was subjected to SDS-10% PAGE. Proteins were electrophoretically transferred onto a nitrocellulose membrane and C3b degradation products were visualized by Western immunoblotting using goat anti-human C3b (1:500; Quidel) and then AP-conjugated mouse anti-goat IgG antibody (1:1000; Santa Cruz Biotechnology, Dallas, Texas, USA). Bands were revealed by adding the AP substrate.

Phagocytosis assays

Activation of THP-1 monocytes was carried out as described by Segura *et al*. with some modifications [43]. Briefly, differentiation of monocytes was carried out by pre-treatment with phorbol 12-myristate 13-acetate (Sigma) (100 ng/mL) for 48 h prior to the test. Following differentiation, cells were washed three times with PBS and medium without antibiotics was added to each well. Bacteria were grown to mid-logarithmic phase and harvested by centrifugation. After incubation, 450 µL of freshly thawed human serum (complement preserved, Quidel) were added to bacteria. Cells were infected with bacterial preparation (10^7 CFU/well, MOI: 10) and incubated at 37 °C in 5% CO_2 for 90 minutes. Optimal incubation time and MOI were chosen based on preliminary studies (data not shown). After incubation,

cell monolayers were washed twice with PBS and incubated for 1 h with medium containing 5 µg/mL penicillin G (Sigma) and 100 µg/mL of gentamicin (Gibco) to kill extracellular bacteria. Cell monolayers were washed three times with PBS and lysed with sterile cold water. Viable intracellular bacteria were determined by plating serial dilutions on THA as described above. Each test was repeated twice in three independent experiments.

Whole blood bacterial killing assay

Whole blood bacterial killing assay was adapted from whole blood culture system as previously described [51]. Blood from three 5 week-old healthy pigs was collected from the jugular vein using Vacutainer Heparin blood collection tubes. Animals originated from a specific pathogen-free herd, which had not presented any isolation of *S. suis* from diseased animals for at least the last 2 years. Blood was then diluted in RPMI 1640 culture medium (Invitrogen, Burlington, ON, Canada) in order to obtain a concentration of 1 x 10^7 leucocytes/mL. Plasma was used as control and similarly processed as the blood sample. Bacteria were grown and washed as described for phagocytosis assays and suspended in RPMI 1640 medium to a concentration of 1 x 10^6 CFU/mL. For killing assay, 250 µL of bacterial preparation were then added to 250 µL of blood mixture. Infected blood cells and reference control (plasma) samples were collected after 2 hours of incubation at 37 °C in 5% CO_2 with manual agitation every 20 minutes. To determine bacterial counts, serial dilutions of infected blood cultures were plated onto THB agar to accurately determine the CFU/mL. The percentage of killed bacteria was calculated as follows: 1-(Bacteria recovered in blood/Bacteria in plasma) x 100%. Each test was repeated twice in three independent experiments.

Statistical analysis

All data are expressed as mean ± SEM. Data were analyzed for significance using the one-way ANOVA test followed by a Dunnett post-hoc test for multiple comparisons or with Student's t-test for comparisons between two groups. A P value < 0.05 was used as a threshold for significance (*). Values < 0.01 were considered as highly significant (**).

Results

Mutant characterization

Inactivation of *fhb* and *cps* genes was previously done and mutants were already characterized, consequently, no further characterization of our equivalent mutants was done [11,13]. In order to further investigate the role of Fhbp in *S. suis* serotype 2, the gene encoding this surface protein [10] was inactivated by a specific in-frame allelic deletion in the wild-type P1/7 strain. Western-blotting using an anti-Fhbp polyclonal antibody depicted in Fig. 1 clearly shows that the resulting Δ*fhbp* mutant does not express Fhbp (Figure 1). Complementation by exogenous Fhbp expression restored the expression of Fhbp. Inactivation of *fhbp* (or previously described inactivation of *fhb* and *cps2F*) had no major consequence on bacterial growth when compared to the wild-type strain (data not shown). Coagglutination and dot-ELISA tests showed that all mutants (with the exception of those including a *cps2F* deletion) were as encapsulated as the wild-type strain (results not shown). Consequently, hydrophobicity tests showed low hydrophobicity (< 5%) for the wild-type strain as well as all mutants, except for those with the *cps2F* deletion (>90%).

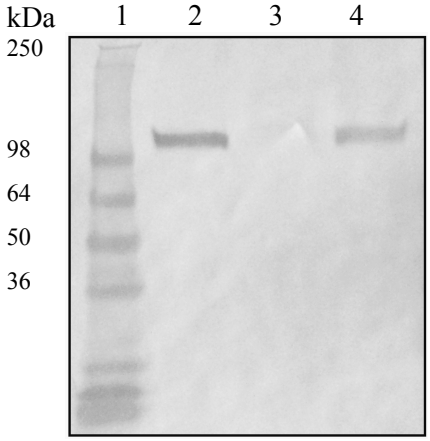

Figure 1. Western-blotting showing Fhbp protein expression in *S. suis* wild-type strain P1/7 and complemented Δ*fhbp* mutant but not in the isogenic Δ*fhbp* mutant. Whole bacteria of *S. suis* wild-type strain P1/7 (lane 2), Δ*fhbp* mutant (lane 3) and complemented Δ*fhbp* mutant (4) were tested for Fhbp expression. Samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Fhbp protein was detected with a monospecific rabbit polyclonal antiserum against Fhbp. Fhbp protein was not detected in Δ*fhbp* mutant whereas a clear positive reaction was obtained for the wild-type strain and the complemented mutant. Molecular weights in kDa are indicated on the left side of the figure.

Adhesion and invasion assays

The interactions (adhesion and invasion) of the *S. suis* wild-type strain (P1/7) as well as mutants defective for factor H binding proteins with human epithelial cells (A549) and human endothelial cells (hBMEC) in presence or absence of human factor H was investigated. As shown in Figures 2A and 2B, adhesion of *S. suis* wild-type strain to A549 human epithelial and hBMEC human endothelial cells was significantly increased in presence of factor H, especially for the epithelial cells (P = 0.006 and P = 0.04, respectively). In contrast, no significant differences were observed regarding invasion of both cell types (Figures 2C and 2D). The role of different factor H binding proteins in such interactions was further investigated. Adhesion levels to hBMEC and A549 cells of single knock-out Δ*fhb* and Δ*fhbp* mutants in the presence of factor H were similar to those obtained with the wild-type strain P1/7 (Figures 3A and 3B). The double knock-out Δ*fhb*/Δ*fhbp* mutant showed a significant decrease in adhesion levels to epithelial cells (P = 0.0279) and to endothelial cells (P = 0.0214) (Figures 3A and 3B). It has to be noted that the double knock out mutant adhered similarly to the wild-type strain to both cell types in absence of factor H (P > 0.05, Figures 3C and 3D).

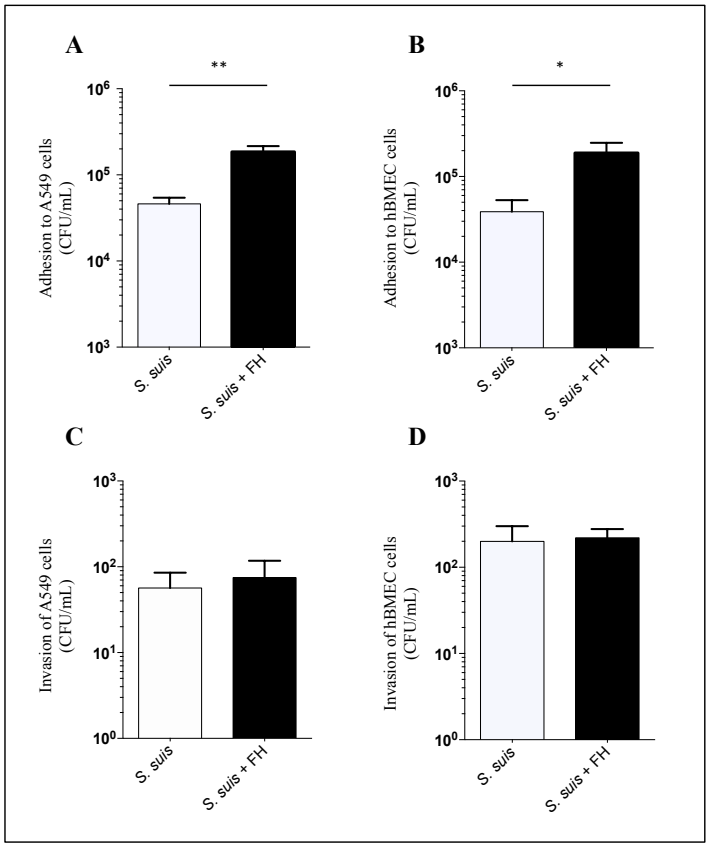

Figure 2. Effect of factor H on cell adhesion and invasion by *S. suis* wild-type strain P1/7. *S. suis* adhesion to (A) human lung epithelial cells A549 and (B) human brain microvascular endothelial cells (hBMEC). Results were determined after 1 h exposure of A549 and hBMEC cells to *S. suis*, followed by extensive washing of non-adherent bacteria and cell lysis to obtain *S. suis* viable counts. Results are expressed as recovered CFU/mL. Significant differences between the wild-type strain P1/7 preincubated with factor H and the same strain preincubated in PBS were observed for both A549 and hBMEC cells (***P* = 0.006 for A549 and **P* = 0.04 for hBMEC), as determined by one-way ANOVA. *S. suis* invasion of (C) human lung epithelial cells A549 and (D) hBMEC. Results were determined after 1 h exposure of cells to *S. suis*, followed by antibiotic treatment to kill extracellular bacteria and by cell lysis to obtain *S. suis* viable counts. No significant differences were observed. Data are expressed as mean ± SEM of at least four independent experiments.

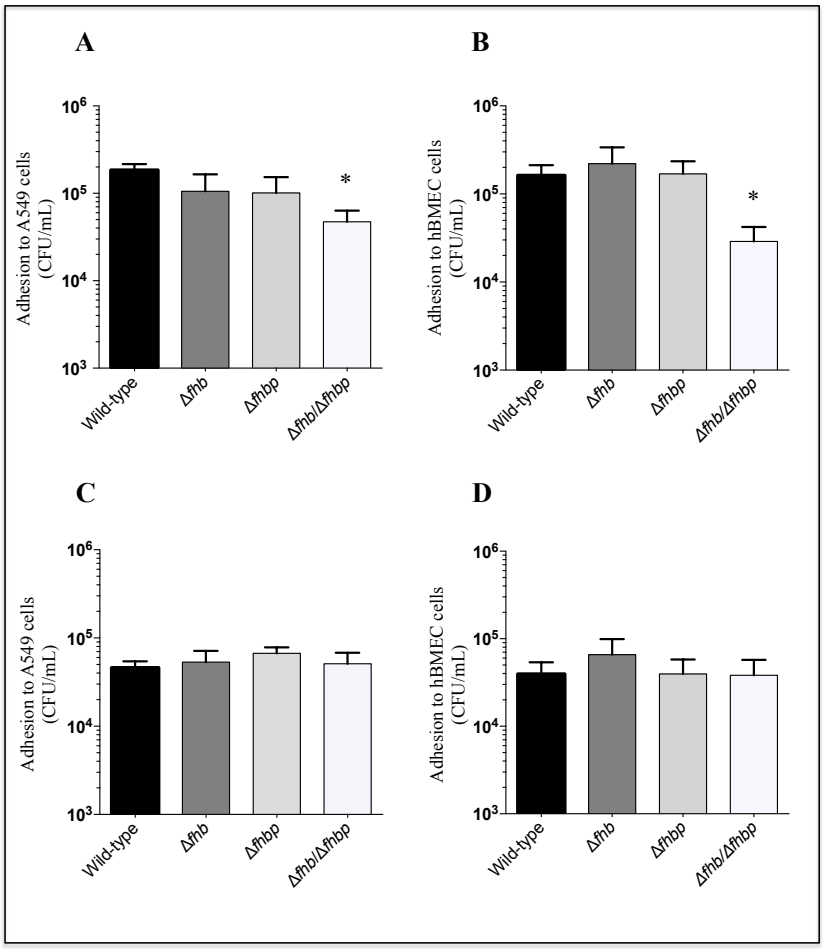

Figure 3. Effect of the deletion of *fhb* and *fhb*p on the *S. suis* adhesion to A549 and hBMEC cells in the presence or absence of factor H. Adhesion of *S. suis* Fhb and Fhbp deficient mutants to (A and C) human lung epithelial cells A549 and to (B and D) human brain microvascular endothelial cells (hBMEC) in presence (A and B) or absence (C and D) of human factor H. Experiments were performed as described in Figure 2. Results are expressed as recovered CFU/mL. Significant differences between the double knock-out Δ*fhb*/Δ*fhbp* mutant and wild-type strain P1/7 as well as single mutants were observed in presence of factor H for both A549 (**P* = 0.0279) and hBMEC cells (**P* = 0.0214), as determined by one-way ANOVA. No significant differences were observed between the wild-type strain P1/7 and single deletion mutants (Δ*fhb* and Δ*fhbp*). Data are expressed as mean ± SEM of at least four independent experiments.

Evaluation of the role of factor H binding proteins on *S. suis* factor H deposition

The deposition of factor H to *S. suis* strains was further evaluated. *Streptococcus mutans* was used as a negative control and showed only a weak deposition of factor H. Surprisingly, no significant differences were observed between the wild-type and *S. suis* mutant strains as depicted in Figure 4. Indeed, they were all similarly able to bind factor H ($P > 0.05$).

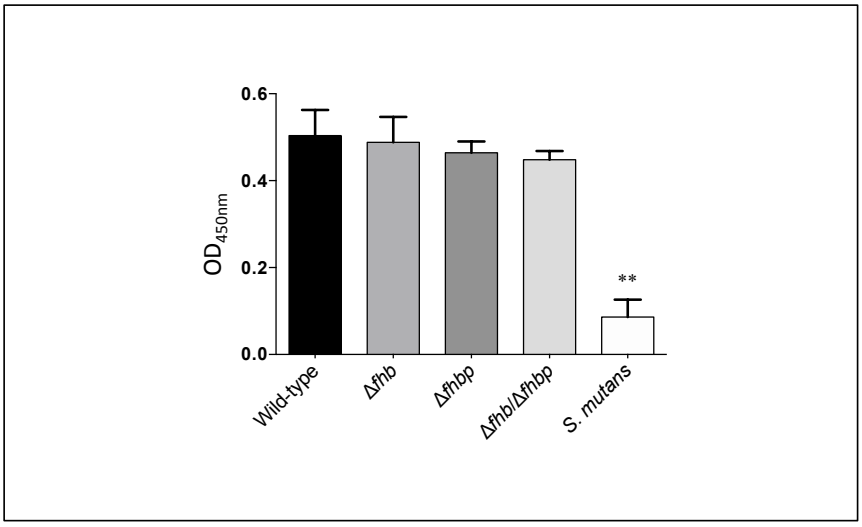

Figure 4. Deposition of factor H to the *S. suis* cell surface: role of Fhb and Fhbp. Deposition of factor H to the bacterial cell surface was detected using an ELISA assay. *Streptococcus mutans* was included as a negative control for factor H binding. There were statistically significant differences between all *S. suis* strains and *S. mutans* as determined by one-way ANOVA (**$P < 0.01$). No significant differences were observed between the *S. suis* wild-type strain P1/7 and isogenic mutants Δ*fhb*, Δ*fhbp* and Δ*fhb*/Δ*fhbp*.

Evaluation of the role of CPS for *S. suis* factor H deposition

Aforementioned results suggest the presence of additional factors involved in factor H recruitment to the surface of *S. suis*, we further evaluated whether the CPS is also able to bind factor H at the bacterial surface. The recruitment of factor H in the absence of CPS was investigated using the non-encapsulated Δ*cps2F* and and the triple Δ*fhb*/Δ*fhbp*/Δ*cps2F* mutants. As shown in Figure 5A, the Δ*cps2F* mutant was significantly affected in its capacity to recruit factor H when compared to the wild-type strain ($P = 0.0376$). Moreover, the additional effect of CPS and factor H binding proteins can be observed with the triple mutant (Δ*fhb*/Δ*fhbp*/Δ*cps2F*), for which significant differences were observed when compared not only to the wild-type strain ($P = 0.0004$) but also to the Δ*cps2F* mutant ($P = 0.0181$).

To confirm that the CPS is able to bind factor H, and to evaluate the importance of the sialic acid moiety in such an interaction, an ELISA test using highly purified native and desialylated

CPS was performed. Both native and desialylated CPS (at a concentration of at least 1 µg/mL) could significantly bind factor H ($P = 0.0006$ and 0.0012, respectively) (Figure 5B).

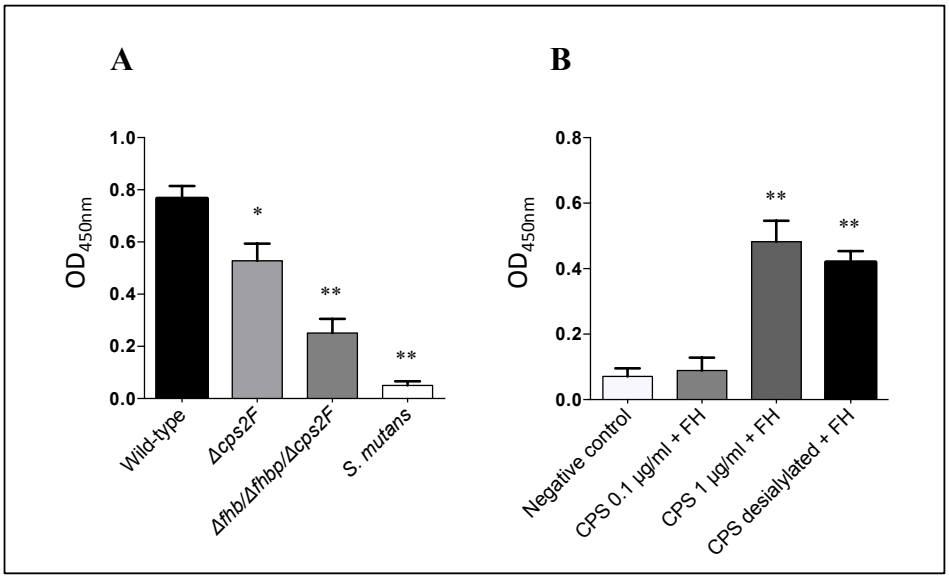

Figure 5. Deposition of factor H to the *S. suis* cell surface: role of CPS and its sialic acid moiety. Results of ELISA showing binding of factor H to (A) non-encapsulated *S. suis* and to (B) *S. suis* purifed CPS. There were statistically significant differences between groups for Figures 5A and 5B as determined by one-way ANOVA. In Figure 5A, significant differences with the wild-type strain are depicted with asterisks (*$P < 0.05$, **$P < 0.01$). Data are expressed as mean ± SEM of at least three independent experiments. In Figure 5B, different concentrations (0.1 and 1 mg/mL) of precoated purified *S. suis* native and desialylated CPS were incubated with factor H (10 µg/mL). Significant differences were observed with factor H incubated with native and desialylated CPS at 1 µg/mL *vs.* control incubated without CPS (**$P = 0.0006$ and **$P = 0.0012$, respectively). No significant differences were observed between native and desialylated CPS in their capacity to bind factor H ($P > 0.05$).

C3b cleavage assay

The functional activity of factor H bound to the surface of *S. suis* was tested using a factor I–cofactor assay. C3b degradation products were identified by SDS-PAGE/Western immunoblotting. As depicted in Fig. 6, all strains tested retained the capacity to degrade C3b as shown by the appearance of the α' 68 kDa cleavage product in addition to the α-chain (116 kDa) and the β-chain (75 kDa) of C3b. Positive control with P1/7 wild-type strain (lane 6) shows normal C3b cleavage product (α' 68 kDa) by factor I in presence of factor H. The 68

kDa band does not appear with the P1/7 strain in the absence of factor H (lane 3; negative control).

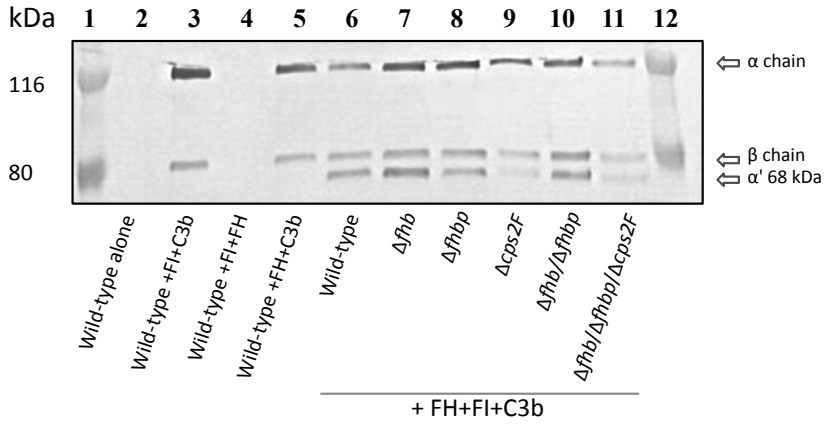

Figure 6. Factor-I cofactor assay showing C3b degradation by *S. suis* strains. Immunoblot shows that bound factor H to *S. suis* strains serves as cofactor for factor I-mediated cleavage of C3b, resulting in formation of an α'68 kDa chain. Lane 1; molecular mass marker, 2: Wild-type strain P1/7 alone; 3: Wild-type strain P1/7+FI+C3b; 4: Wild-type strain P1/7+FI+FH; 5: Wild-type strain P1/7+FH+C3b; 6: Wild-type strain P1/7+FH+FI+C3b; 7: Δ*fhb* mutant strain+FH+FI+C3b; 8: Δ*fhbp* mutant strain+FH+FI+C3b; 9: Δ*fhb*/Δ*fhbp* mutant strain+FH+FI+C3b; 10: Δ*cps2F* mutant strain+FH+FI+C3b; 11: Δ*fhb*/Δ*fhbp*/Δ*cps2F* mutant strain+FH+FI+C3b; 12: molecular mass marker. All strains retained the capacity to bound factor H in a way that serves as cofactor for factor I-mediated cleavage.

Phagocytosis assay

We investigated the role of both Fhb and Fhbp on the resistance capacity of *S. suis* to complement-mediated opsonophagocytosis. As shown in Figure 7, no significant differences were observed when comparing the wild-type strain with the single or double Δ*fhb*/Δ*fhbp* mutant. In contrast, the non-encapsulated strain Δ*cps2F,* used as control, was susceptible to phagocytosis by macrophages showing significant difference with both wild-type and mutants strains ($P < 0.0001$) (Figure 7).

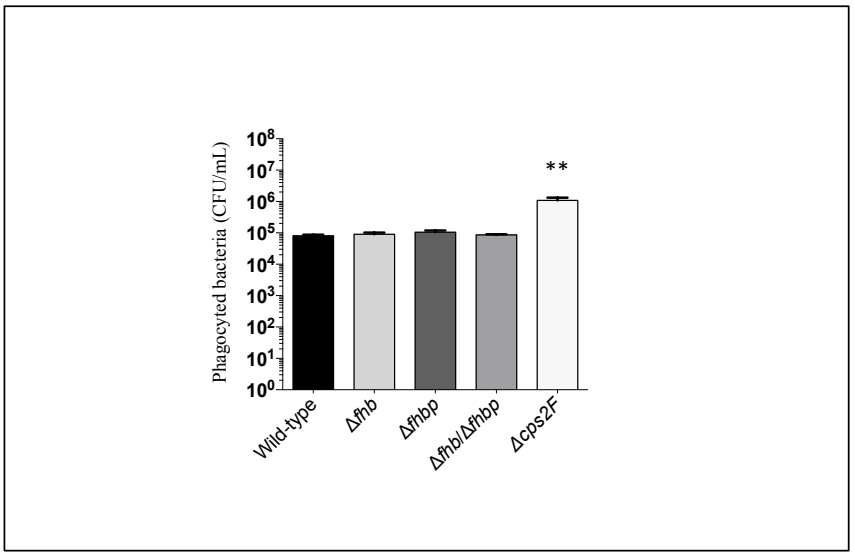

Figure 7. Phagocytosis of *S. suis* strains by THP-1 human macrophages in presence of complement-rich serum. Bacteria (1 x 10^7 CFU/mL) were incubated for 90 min with cells (MOI=100) in presence of human serum, followed by gentamicin/penicillin G treatment to kill any remaining extracellular bacteria after incubation. Intracellular counts were done after three washes and cell lysis with water. Results represent the mean (CFU/mL) ± SEM of four independent experiments. There were not statistical differences between the *S. suis* wild-type and any of the factor H-binding protein mutants. The non-encapsulated mutant (positive control) was significantly more phagocytosed as determined by one-way ANOVA (**$P < 0.01$).

Whole blood bacterial killing assay

Bacterial killing by whole swine blood showed no differences between the wild-type strain P1/7 and either of the Δ*fhb,* Δ*fhbp* or Δ*fhb*/Δ*fhbp* mutants (Figure 8). Percentage of killing varied from 40 to 60% for all strains. Only the non-encapsulated Δ*cps2F* mutant strain was shown to be highly susceptible, as expected, with 90% of bacteria being killed (Figure 8).

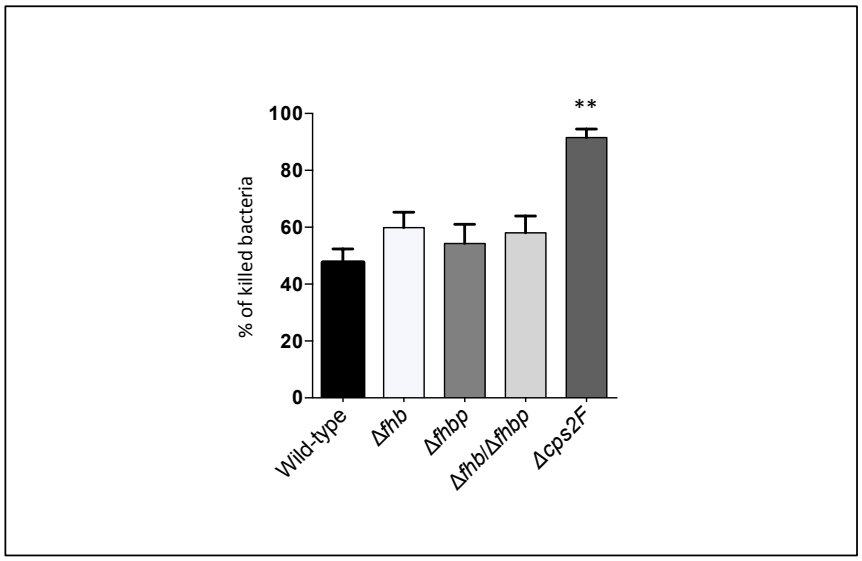

Figure 8. Killing of *S. suis* by swine whole blood cells. Bacteria (5 x 10^5 CFU) were incubated for 120 min with swine whole blood or with blood serum (bacteria alone). The percentage of killed bacteria was calculated as follows: 1-(Bacteria recovered in blood/bacteria recovered in serum) x 100%. Data are expressed as mean ± SEM of at least three independent experiments. There were not statistical differences between the *S. suis* wild-type and any of the factor H-binding protein mutants. The non-encapsulated mutant (positive control) was significantly more killed as determined by one-way ANOVA (**$P < 0.01$).

Discussion

The role of complement during the systemic infection caused by *S. suis* is still controversial. In an early report, Brazeau *et al.* suggested a limited role of complement in phagocytosis and killing of well encapsulated *S. suis* [14]. More recent studies showed that the complement limits *S. suis* invasion when using an intranasal mouse model of infection [15]. However, the role of complement in the *S. suis* systemic infection still remains to be confirmed.

Factor H is the key fluid phase regulator of the alternative complement pathway and acts as a cofactor in the factor I-mediated proteolysis of C3b [16]. Hence, pathogenic bacteria have developed mechanisms to recruit factor H to their surface to avoid complement attack and opsonophagocytosis. Indeed, several pathogens possess adhesins that are able to recruit factor H to their surface to degrade C3b in iC3b in order to reduce opsonophagocytosis by host cells [17,18]. Recruitment of factor H may also help bacteria to adhere and to invade epithelial and

endothelial cells [19]. For most pathogens, different proteins expressed at the bacterial surface are responsible for factor H deposition [20]. In the case of *S. suis*, two factor H-binding proteins (Fhb and Fhbp) have been reported and characterized so far [10,11]. In the present study, we investigated the role of factor H, as well as those played by Fhb and Fhbp, as adhesion/invasion molecules for *S. suis* during bacterial interaction with host cells. In addition to these proteins, the role of CPS in the recruitment of factor H was also studied.

Results showed that factor H acts as an enhancing adhesion molecule for *S. suis* to epithelial and, to a lesser extent, endothelial cells. This function had been previously described for other human pathogen, including *S. pneumoniae* [19]. It has been suggested that factor H binds to pneumococci, and bound factor H is oriented in a way that it can interact with polyanionic molecules (glycoaminoglycans) on the surface of host cells [19]. Receptors used by the factor H bound to the *S. suis* surface in order to promote bacterial adhesion are still unknown. The fact that the increased adhesion was considerably more significant with epithelial cells may indicate that this mechanism could mainly benefits bacteria during the early steps of the infection. Interestingly, although single Δ*fhb* and Δ*fhbp* mutants adhered similarly to the wild-type strain, a slight but significant reduction in adhesion to both cell types was observed with the double Δ*fhb*/Δ*fhbp*, suggesting, to a certain extent, an additive role when the two proteins are present. However, the possibility that the inactivation of one *S. suis* factor H binding protein (Fhb or Fhbp) induces the overexpression of the second protein (Fhbp or Fhb, respectively) cannot be ruled out. The actual role *in vivo* of factor H-dependent increased adhesion should be confirmed. In contrast to what has been described for *S. pneumoniae* [19], factor H does not increase the invasion properties of *S. suis* to the epithelial or endothelial cells tested. As expected, the absence of Fhb and Fhbp did not influence host cell invasion.

Interestingly, the bacterial proteins evaluated in this study do not play a critical role in adhesion/invasion to cells *per se*, since Δ*fhb*, Δ*fhbp* single mutants as well as the double Δ*fhb*/Δ*fhbp* mutant behave similarly to the wild-type strain in the absence of factor H. These results were unexpected, since the pneumococcal surface protein C (PspC), which presents homology to the *S. suis* Fhbp, directly participates in *S. pneumoniae* cell adhesion by binding to host glycoconjugates and sialic acid residues [21]. In addition, the Fhb protein has been

reported to be responsible for the *S. suis* binding to Galα1-4Gal present in glycolipid Gb03—abundant in epithelial and endothelial cells [22,23]. We could not find any previous work indicating that the Gb03 is precisely present in the epithelial cell line A549. However, this glycolipid has previously been shown to be present in the hBMEC line used in this study [24]. If the factor H-binding proteins of *S. suis* do not play any role in adhesion to epithelial and endothelial cells or if such a hypothetical role is redundant due to the presence of additional adhesins remains to be studied.

Surprisingly, although both Fhb and Fhbp proteins together play a limited but certain role in factor H-mediated adhesion to cells together, the absence of either one or both of these proteins had no effect on the overall capacity of *S. suis* to recruit factor H at the bacterial surface. It might be possible that, although similar amounts of factor H may be recruited to the bacterial surface by other factor H binding factors, specific binding of factor H to both studied proteins may induce steric modifications that could slightly modify the enhanced capacity of bacteria to adhere to host cells. It has been previously shown that very specific domains of factor H are involved in the interactions with factor H-binding proteins [25]. However, this hypothesis remains to be elucidated.

Since factor H is still recruited at the bacterial surface of factor H-binding protein defective mutants, we hypothesized that additional bacterial factors may play important roles in such activity. It has been reported that some sialylated pathogens are able to recruit factor H due to the presence of sialic acid at their surface, as it is the case of *Neisseria gonorrhoeae* and *Pasteurella aeruginosa* [26,27]. Since the CPS of *S. suis* serotype 2 is rich in sialic acid [28], the capacity of such a CPS to interact with factor H was evaluated. To carry out such studies, a capsule-deficient (*Δcps2F*) mutant and a triple mutant deficient in both factor H binding proteins and the CPS (*Δfhb/Δfhbp/Δcps2F*) were produced. Interestingly, the non-encapsulated mutant recruited significantly less factor H to the bacterial surface than the wild-type strain. Although the CPS is thought to limit the exposition of cell surface components [29], it does not seem to be the case for factor H binding proteins. It has also been suggested for other pathogens that the presence of CPS does not reduce surface availability of factor H binding proteins [30,31]. Indeed, a clear collaborative and additive factor H-recruitment activity

between Fhb and Fhbp proteins together with the CPS can be suggested, since the triple mutant (*Δfhb/Δfhbp/Δcps2F*) recruited significantly less factor H deposition at the surface than both, the wild-type and the double-mutant (*Δfhb/Δfhbp*) mutant.

Marques *et al*. (1992) demonstrated that wild-type Group B *Streptococcus* binds lower levels of active C3b as compared to mutants deficient in capsule and sialic acid expression, postulating that this could be due to the binding of factor H to sialic acid [32]. However, the sialic acid mutant used in that particular study was poorly encapsulated [33] and differences observed could have been the result of the absence of sialic acid, CPS or both. A function of the CPS in resistance to complement deposition has already been shown for *S. suis* [13]. In addition, it has been recently demonstrated that the CPS would play an important role in the protection against the complement system in a *S. suis* experimental mucosal infection model [15]. In this study, and for the first time, we report that the CPS from a Gram-positive pathogen is able to recruit factor H. Desialylated CPS was, unexpectedly, also able to bind similar amounts of factor H than the native CPS, indicating that sialic acid is not the main sugar involved in binding such host factor. An encapsulated but sialic-acid negative mutant cannot be used to confirm these results, since such mutants are either non-encapsulated [33,34] or lethal [35] for *S. suis*. The mechanisms by which the CPS binds the factor H, as well as the ability of CPS from different *S. suis* serotypes to bind factor H remain to be elucidated. It has been previously shown that different serotypes of *S. pneumoniae* were able to differently recruit human factor H to their cell surface [30], although the specific role of the CPS was never investigated. Finally, the role of factor H interactions with a non-encapsulated *S. suis* strain on bacterial adhesion studies could not be performed, since capsule deficient mutants already adhere at very high levels to cells [36].

As mentioned, the triple (*Δfhb/Δfhbp/Δcps2F*) mutant is still able to bind factor H to a certain extent, as compared to the *S. mutans* used as negative control. In addition, the wild-type strain of *S. suis* as well as all mutant strains tested in the factor-I cofactor assay equally retained the capacity to degrade C3b in the presence of factor H. These results strongly suggest the presence of additional *S. suis* surface components able to recruit factor H. For example, Shao *et al*. (2011) identified an immunogenic cell surface-associated protein (histidine triad protein

of *S. suis*) involved in evasion of complement-mediated host innate immune responses by preventing C3 deposition on the bacterial surface of *S. suis* by so far unknown mechanisms [37]. Hence, other proteins redundantly being able to bind factor H may also exists.

Results obtained with the factor-I cofactor assay were confirmed by phagocytosis and killing assays. Single as well as the double *Δfhb/Δfhbp* mutants were as resistant to phagocytosis as the wild-type strain. This is the first time that the Fhbp is evaluated. However, these results are in disagreement with those previously published with a Fhb deficient mutant derived from a highly virulent Chinese strain [11], where the authors showed that the Fhb mutant was highly susceptible to phagocytosis by human neutrophils [11]. In addition, although that study also reported that the *Δfhb* mutant was sensitive to the blood-killing test and was less virulent in pigs (with lower levels of bacteremia) [11], no differences could be observed with the whole cell blood-killing test in the present study. Besides some technical details in the methodology as well as the use of different strains, we do not have clear explanations to explain such differences; in our hands, both Fhb and Fhbp proteins do not seem to be highly involved in factor H recruitment, resistance to phagocytosis or killing, and it would be highly surprising that these proteins play a critical role in virulence. In fact, single and double-factor H binding proteins mutants used in the present study were shown to be as virulent as the wild-type strain in a mouse model of infection (unpublished). Finally, the role of the CPS in phagocytosis and killing has largely been shown [13,34], and it is probably not linked to the factor H recruitment activity.

In conclusion, binding of factor H to the *S. suis* surface increases bacterial adhesion to host cells, especially epithelial cells, and allows degradation of C3b. This factor H recruitment seems to modestly occur through Fhb, Fhbp and the CPS. However, these factors do not seem to be critical for such factor H binding activity as well as its consequences. It seems that recruitment of factor H to *S. suis* surface is multifactorial and redundant.

Conclusions

Results obtained in this study showed a role of factor H in the adhesion of *S. suis* to epithelial and, to a lesser extent, endothelial cells. Both Fhb and Fhbp binding proteins play a certain role in such increased factor H-dependent adhesion. None of these proteins were a critical adhesin *per se* (in the absence of factor H). The CPS, independently of the presence of its sialic acid moiety, was also shown to bind factor H. The absence of both factor H-binding proteins does not influence the resistance of *S. suis* to phagocytosis by human macrophages and to bacterial killing by swine blood. It seems that recruitment of factor H to *S. suis* surface is multifactorial and redundant.

Acknowledgments

Katy Vaillancourt is acknowledged for her technical assistance. This study was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC, grant #154280 to Marcelo Gottschalk, with a collaboration of NSERC grant #342150 to Mariela Segura and NSERC grant #04146 to Daniel Grenier. David Roy is the recipient of a scholarship from the Swine and Poultry Infectious Disease Research Center.

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ARTICLE VI

The protective protein Sao (surface antigen one) is not a critical virulence factor for *Streptococcus suis* serotype 2

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Publié dans: Microb. Pathog. **2014**. 67–68, 31–35. doi: 10.1016/j.micpath.2014.02.002.

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 et à la réalisation des expériences, à l'analyse des résultats et à l'écriture de l'article.

Abstract

Surface antigen one (Sao) is protein that was shown to protect pigs in vaccination trials against disease caused by *Streptococcus suis*. Here, we used an isogenic Δ*sao* mutant to investigate the insofar unknown role of Sao in *S. suis* virulence. The Δ*sao* mutant and its wild-type parent strain (sequence type 1, European strain) did not present significant differences on levels of adhesion/invasion of swine respiratory epithelial cells. Moreover, the Δ*sao* mutant resisted phagocytosis by macrophages similarly to the parent strain and it was still virulent in a mouse model of infection. Our findings suggest a minor role of Sao in *S. suis* serotype 2 virulence.

Introduction

Streptococcus suis is a major agent of meningitis and septicemia in swine and is also an emerging zoonotic pathogen [1]. *S. suis* is now considered one of the most important causes of human adult meningitis in Southeast Asian countries [2, 3]. Extensive research during the last two decades has identified many factors involved in the virulence of this pathogen including notably the capsular polysaccharide (CPS), which plays a major protective role against host immunity [4]. However, there is still no validated method to predict whether a strain is potentially virulent. Some virulence markers (without clear explanation of their roles in the pathogenesis of the infection) might be used in some parts of the world, such as the muramidase-released protein and the extracellular protein factor [5]. However, it is still impossible to ensure that a strain without these factors is, indeed, avirulent [6]. In fact, there is probably not a universal virulence factor for *S. suis* serotype 2 strains [6].

Prevention of *S. suis* disease in swine by means of vaccination is a long-sought goal. Vaccination is still carried out exclusively with bacterins in the field, with usually high rate of failure [1], although a few studies showed homologous protection [7]. Different subunit vaccine candidates have intensively been tested during the last years, using different protocols and adjuvants [4, 6]. Surface antigen one (Sao) was identified by screening of a phage display library using convalescent swine sera [8]. Sao is a protein possessing the consensus sequence LPXTG and therefore is anchored to the cell-wall peptidoglycan by the housekeeping sortase A

and it has been considered to be an interesting protective immunogen by different research groups [9-11].

Despite encouraging results in immunization trials, the biological role of Sao has remained so far elusive. In this study, *in vitro* and *in vivo* results obtained with an isogenic Δ*sao* mutant suggest a minor contribution of Sao to the full virulence of *S. suis* serotype 2.

Materials and Methods

Bacterial strains and growth conditions

The well-characterized *S. suis* serotype 2 virulent strain 31533 (sequence type 1) was used as wild-type strain [12, 13]. A non-encapsulated isogenic mutant (strain B218, obtained from the same wild-type strain) was used as a positive control for the phagocytosis study (see below) [13]. An isogenic Δ*sao* mutant was constructed by the procedures described below. Bacteria were cultured as previously reported [14]. When needed, antibiotics (Sigma, Oakville, ON, Canada) were added to the culture media at the following concentrations: for *S. suis*, spectinomycin at 100 µg/ml and chloramphenicol (Cm) at 5 µg/ml; for *Escherichia coli*, kanamycin and spectinomycin at 50 µg/ml and Cm at 30 µg/ml.

DNA manipulations

S. suis genomic DNA was prepared by the guanidium thiocyanate method [15]. Minipreparations of recombinant plasmids and transformation of *E. coli* were performed by standard procedures [16]. Restriction enzymes and DNA-modifying enzymes were purchased from Fisher (Ottawa, ON, Canada) 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 Life Technologies (Burlington, ON, Canada).

Construction of the isogenic Δsao mutant

Regions upstream and downstream of the *sao* gene were amplified by PCR using genomic DNA obtained from *S. suis* serotype 2 strain 31533. Primers (introducing restriction sites, underlined in sequence) were: Sao-upF (5'-GCTCTCGAAAGCTTGCTTCCTTGCGT-3', HindIII) and Sao-upR (5'-AGAGCAACAGATCTAAATAATAC-3', BglII) and Sao-dwF (5'-GAAACTGCTAAGCAGATCTTACCAGTA-3', BglII) and Sao-dwR (5'-GGCATCAAGGATCCGGAAGATTTTG-3', BamHI), respectively. After digestion with BglII, PCR amplicons were fused giving rise to the Δ*sao* allele which was then cloned into vector pCR4 (Life Technologies). A DNA segment containing the Cm resistance-encoding gene *cat* was then amplified from plasmid pSET5s [17] using primers catF (5'-ACCGAACTAGATCTTGATGAAAA-3, BglII) and catR (5'-CAGAAAAAGAAAGATCTGGATCT-3', BglII), digested with BglII and cloned into the BglII site of the Δ*sao* construct. This new construct was then subcloned into the HindIII/BamHI sites of the thermosensitive *E. coli-S. suis* shuttle plasmid pSET4s [17]. Electroporation of *S. suis* strain 31533 with the knockout vector and procedures for isolation of mutants were those described previously [18]. Allelic replacement was confirmed by PCR and DNA sequencing analysis. Amplification products were purified with the QIAgen PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced with an ABI 3730xl automated DNA sequencer, using the ABI PRISM dye terminator cycle version 3.1 (Applied Biosystems, Carlsbad, CA, USA).

Western blotting

Supernatants of 10 ml overnight cultures of the strains under investigation were harvested by centrifugation. After concentration (10-fold) with a Thermo savant DNA120 speedvac (Thermo Fisher Scientific, Waltham, MA, USA), supernatants were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels. Western-blotting was carried out as previously described [8], using supernatant of a hybridoma culture producing monoclonal antibodies specific to Sao protein of *S. suis*.

S. suis ***adhesion to and invasion of porcine tracheal cells***

The pig trachea epithelial cell line (NPTr) was used and cultured until confluence as previously described [14]. Cells were infected with *S. suis* (10^6 CFU/well, MOI:10) and incubated at 37°C in 5 % CO_2 for different incubation times (see below). The invasion assay was performed by the antibiotic protection assay as previously described [14]. Levels of invasion were expressed as the total number of CFU recovered per well. An "adhesion assay" which in fact quantifies total cell-associated bacteria (intracellular bacteria and surface-adherent bacteria) was performed as previously described [14]. At different incubation times (see results), the levels of "adhesion" (total associated bacteria) were expressed as the total number of CFU recovered per well. Each invasion and adhesion test was repeated at least four times in independent experiments.

S. suis ***phagocytosis assay***

J774-A1 macrophages (ATCC TIB 67, Rockville, MD, USA.) were used for *S. suis* phagocytosis studies as previously described [19]. Macrophages (10^5 cells/ml) were infected with *S. suis* strains (31533, Δ*sao* mutant and non-encapsulated strain B218) at a concentration of 1 x 10^7 CFU/ml (MOI:100) diluted in complete cell culture medium consisting of DMEM supplemented with 10% fetal bovine serum (FBS) (Life Technologies). After different incubation times (see results), the antibiotic protection assay was performed as previously described [19]. Each test was repeated at least four times in independent experiments, and the number of CFU recovered per well was determined.

S. suis ***in vivo infection***

A well-standardized model of murine infection was used [12]. All experiments 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. A total of 33 six-week-old CD1 mice (Charles River Laboratories, Wilmington, MA, USA), divided in 3 groups (sham-inoculated, 31533 and Δ*sao*) of 11 animals each were included in the study. One ml volume of a bacterial suspension (at 1 x 10^7 CFU/ml) or the vehicle solution (THB sham-inoculated

group) was administrated by intraperitoneal injection. Mice were monitored for mortality and clinical signs of sepsis, such as depression, swollen eyes, rough hair coat, lethargy, and nervous signs of meningitis [12]. Blood samples were collected from the tail at 24 and 48 post-infection (p.i.) and plated onto blood agar plates as described [12]. In order to verify if dissemination in organs was different between the wild-type strain and the Δ*sao* mutant, bacterial colonization levels of the spleen was measured in a separate trial, using the same conditions described above. The spleen (0.05 g/organ) was trimmed, placed in 500 µl of PBS (pH 7.3) and homogenized with a vortex. Then, 50 µl of 10^{-2} and 10^{-4} dilutions of the homogenate in PBS was plated onto blood agar plates and bacterial counts evaluated as described above [12]. Only live animals were included in this part of the study to avoid post-mortem contamination of already dead animals.

Statistical analysis

All data are expressed as mean ± SE. For *in vitro* experiments data were analyzed for significance using ANOVA 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.

Results

Inactivation of Sao *in* S. suis *serotype 2*

In order to investigate the role of Sao in *S. suis* serotype 2 virulence, the gene encoding this surface protein was inactivated via a double-cross-over event in a highly virulent wild-type strain (strain 31533), which possess a Sao protein variant of intermediate molecular weight (Sao-M) [9, 20]. Western-blotting using an anti-Sao monoclonal antibody (Fig. 1) and a monospecific polyclonal antibody (results not shown) clearly showed that the resulting Δ*sao* mutant did not express Sao. Inactivation of *sao* had no major consequences on growth either in normal laboratory medium or in the presence or absence of 10% porcine or fetal bovine sera. In addition, the mutant was normally typed as serotype 2 by using the conventional coagglutination test (data not shown) [1].

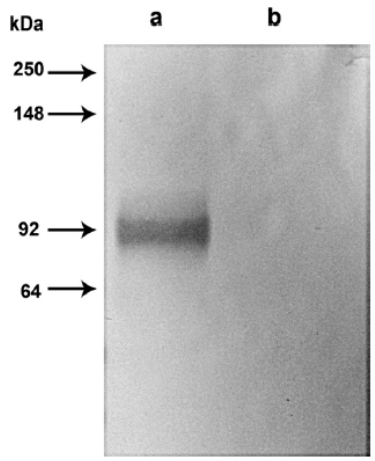

Figure 1. Western-blotting showing Sao protein expression in *S. suis* 31533 wild-type strain but not in its isogenic Δ*sao* mutant. Culture supernatant of *S.suis* wild-type strain 31533 (lane a) and Δ*sao* mutant (lane b) were separated by SDS-polyacrylamide gel electrophoresis and transfered to a nitrocellulose membrane. Sao protein was detected with a specific monoclonal antibodies against Sao. Sao protein was not detected in Δ*sao* mutant supernatant whereas a clear positive reaction was obtained for the wild-type strain. Molecular weights in kDa are indicated on the left side of the figure.

Inactivation of sao *does not influence interactions of* S. suis *with respiratory epithelial cells.*

When the interaction of *S. suis* with epithelial cells were tested *in vitro* under controlled conditions, no significant differences between the Δ*sao* mutant and its parent wild-type strain at different incubation times were found (Fig. 2A and Fig. 2B). These results strongly support the hypothesis that Sao is not a critical factor for *S. suis* serotype 2 adhesion to and invasion of swine tracheal epithelial cells.

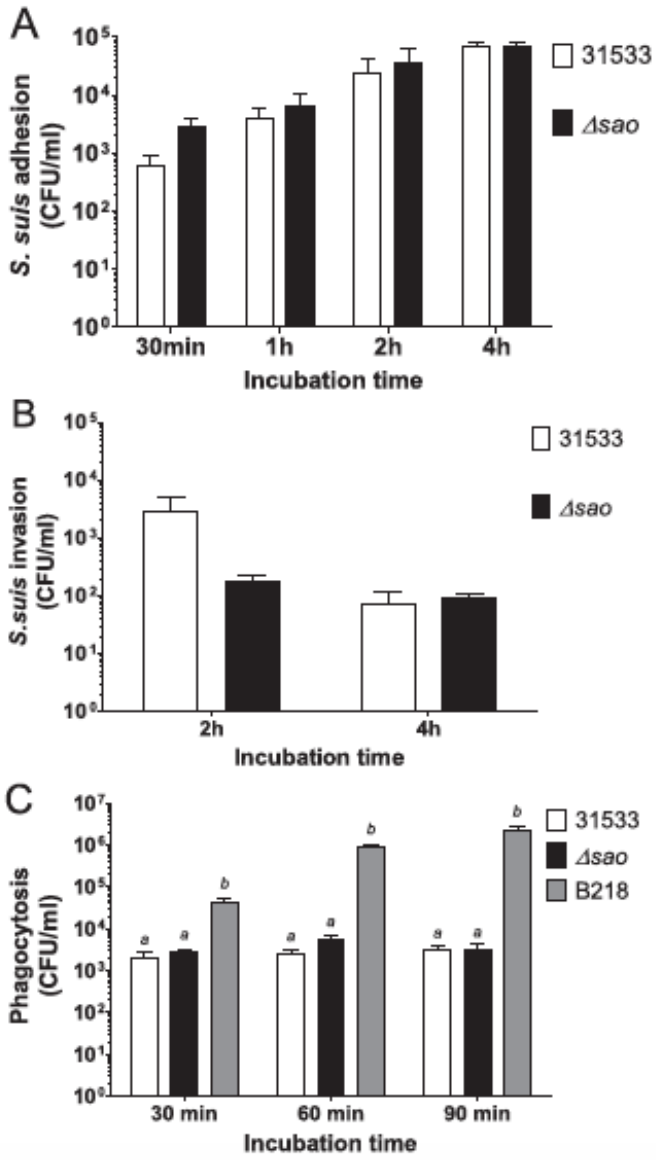

Figure 2. Effect of the deletion of Sao on the adhesion to and invasion of NPTr cells by *S. suis* and on the resistance of *S. suis* to phagocytosis by macrophages. (A) Kinetics of adhesion of the wild-type *S. suis* strain 31533 and the Δ*sao* mutant derivative to NPTr cells. Results were determined after exposure of NPTr cells to *S. suis*, followed by extensive washing of non-adherent bacteria and cell lysis to obtain *S. suis* viable counts. (B) Invasion of wild-type *S. suis* strain 31533 and the Δ*sao* mutant derivative to NPTr cells at two different incubation times. Results were determined by quantitative plating after antibiotic treatment to kill extracellular bacteria. (C) Phagocytosis assay with J774 macrophages at different incubation times. Wild-type *S. suis* strain 31533 and the Δ*sao* mutant derivative were incubated with macrophages. The non-encapsulated B218 mutant was used as a positive control. Numbers of internalized bacteria were determined by quantitative plating after antibiotic treatment to kill extracellular bacteria, and the results are expressed as recovered CFU/ml. Significant differences between the non-encapsulated B218 strain (identified as "*b"*) and both the wild-type strain and Sao deficient mutant (identified as "*a"*) were observed ($P < 0.05$). No significant difference was observed within group "*a"*. Data are expressed as mean ± SEM of at least four independent experiments.

***Resistance to phagocytosis by macrophages is unaffected by inactivation of* sao.**

The effect of the inactivation of *sao* on the resistance to phagocytosis was then evaluated. Fig. 2C shows that both the wild-type strain 31533 and the Δ*sao* mutant were relatively resistant to phagocytosis. Very low numbers of bacteria were recovered from cells and the number of internalized bacteria was constant during different incubation times. In contrast, the control strain (a non-encapsulated mutant) was significantly more phagocytosed by macrophages than either the wild-type strain or Δ*sao* mutant ($P < 0.05$) and number of phagocytosed bacteria increased with longer incubation times (Fig. 2C).

Deletion of* sao *does not impair virulence of* S. suis *in a mouse model of infection.

Results revealed that Sao is not a critical virulence factor for *S. suis*. Indeed, while sham-inoculated animal did not present clinical signs, most mice in groups infected with either the wild-type strain or the Δ*sao* mutant presented severe clinical signs associated with septicemia, such as depression, swollen eyes, rough hair coat, prostration and weakness during the first 72 h p.i. Twenty percent of animals died or were euthanized for ethical reasons within 96 h (Fig. 3A). *S. suis* could be isolated from blood samples of both groups at a high similar bacterial load during the first 48h (Fig. 3B). No significant differences in bacterial concentration in spleen at 48h p.i. were observed between the two strains (data not shown). Clinical sings of meningitis were also observed in both groups between days 4 and 7 p.i., with an identical total mortality of 55% (Fig. 3).

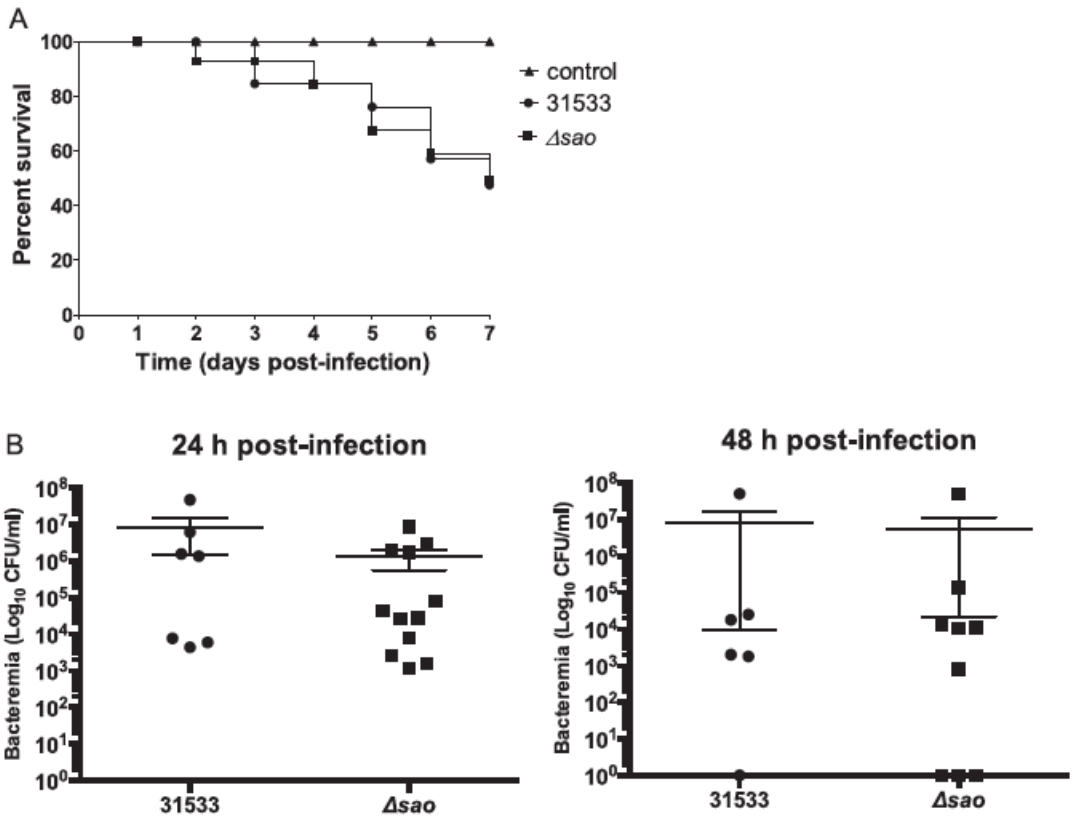

Figure 3. Effect of the deletion of Sao on the virulence of *S. suis*. (A) Survival of mice infected either with the wild-type *S. suis* 31533 strain or the Δ*sao* mutant derivative. No significant differences were observed between groups ($P > 0.05$). In both groups, 55% of the mice died from septicemia or meningitis within 7 days. Control animals remained unaffected. (B) Blood bacteremia at 24h and 48h post-infection with either the wild-type *S. suis* 31533 strain or the Δ*sao* mutant derivative. Data of individuals are presented including geometric mean with 95% confidence interval. No significant differences (P>0.05) between the two groups were observed as determined by ANOVA.

Discussion

S. suis serotype 2 is a major swine pathogen and a zoonotic agent of increasing importance [1]. So far, different genes have been identified as critical for *S. suis* virulence [6]. In addition, certain proteins have been described as being protective, although their role in virulence has not been evaluated [4, 6]. We have previously identified the Sao protein, which is present in most *S. suis* serotypes and tested field strains of serotype 2 [8]. Convalescent-phase swine sera present high titers of antibody against this protein, suggesting that Sao is a potent immunogen expressed during *S. suis* infection. In addition, Sao induced protection in mice and pigs when used with an adjuvant that drives a Th1-type immune response [9]. These findings have suggested that Sao is an interesting candidate to be used in a subunit vaccine [11]. This relatively detailed understanding of the protective capacities of Sao contrasts with the almost non-existent knowledge on its biological role. In this study, we attempted to provide insights into the role of Sao in *S. suis* virulence.

It is still unknown how virulent *S. suis* usually found in very low quantities in tonsils of pigs, manages to cross the first natural line of the host defense to initiate disease. The current most accepted hypothesis is that the pathogen breaches the mucosal epithelium in the upper respiratory tract of pigs [6]. Since Sao is a cell-wall anchored protein, we hypothesized that it may contribute to adhesion and invasion of epithelial cells of the host which are associated with the very first steps of colonization by mucosal pathogens. However, our results showed that Sao does not play a critical role as an adhesin or invasin of respiratory epithelial cells. Indeed, the Δ*sao* mutant presented similar levels of adhesion/invasion than the wild-type strain. It must be noted, however, that it is possible that different surface proteins have similar and redundant functions, and thus an eventual collaborative role of Sao in the first steps of the infection cannot be completely ruled out.

Once in the bloodstream, *S. suis* must be able to resist phagocytosis and killing to keep high levels of bacteremia. It has been proved that the CPS plays a critical role as anti-phagocytic factor [6]. In the present study, a mutant lacking the Sao protein was as resistant to phagocytosis as the wild-type strain, suggesting that Sao is not a major anti-phagocytic factor,

at least in the presence of the CPS. Since non-encapsulated mutants are well phagocytosed, the specific role of Sao protein as anti-phagocytic factor in the absence of CPS cannot be easily evaluated. Due to the low number of bacteria, it was not possible to evaluate survival of *S. suis* organisms phagocytosed. Therefore, an hypothetical role of Sao in intracellular survival cannot be ruled out. Interestingly, antibodies against Sao protein have been described as being opsonic and able to increase phagocyte killing. It is possible that a well expressed surface protein may induce opsonic antibodies without being a virulence factor. The capacity of the Δ*sao* mutant to survive in blood was further confirmed with the experimental infection in a standardized mouse model. In fact, the mutant was able to survive and proliferate in the bloodstream and to colonize organs, inducing disease and mortality similarly to the wild-type strain.

There are other examples of protective proteins present in highly virulent as well as in less virulent serotypes of *S. suis* [21, 22]. In addition, proteins induced by all virulent and non-virulent members of a bacterial species (such as housekeeping proteins) have also been demonstrated to induce protective antibodies. In *S. suis*, the enolase and the 6-phosphogluconate dehydrogenase have both been described as protective antigens [23, 24]. Moreover, live non-virulent *S. suis* strains have also been shown to induce protection against a virulent strain, indicating that some antigens may be protective without being critical virulence factors [25]. Data presented here advocate that Sao, although being a protective antigen, is not a critical virulence factor. It is important to note that the wild-type strain used in the present study is a virulent strain isolated in Europe [12, 13]. This strain presents a typical European profile with a sequence type 1 as tested by multilocus sequence typing (unpublished observations). Strains of *S. suis* serotype 2 isolated from different geographical origins may possess different virulence factors [6]. The possibility that Sao plays a certain role in virulence in serotype 2 strains from other phenotypic and genetic background cannot be ruled out. Further studies to elucidate the fine biological role of Sao are needed.

Acknowledgements

The authors are very grateful to Dr. Daisuke Takamatsu and Dr. Tsutomu Sekizaki for providing the pSET4s and pSET5s plasmids. This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) grant #154280 to MG.

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IV. DISCUSSION

Les composants exprimés en surface représentent des facteurs de virulence importants qui sont susceptibles d'influencer la virulence d'une souche[26]. La CPS et les protéines de surface sont des facteurs exprimés à la surface de la bactérie ayant des rôles importants dans les premières étapes de l'infection, notamment dans la colonisation et la résistance au système immunitaire de l'hôte[26]. La CPS (α2,6 sialylée) est un facteur antiphagocytaire critique pour *S. suis* sérotype 2 et intervient également dans la modulation de la réponse de l'hôte[49, 51]. Quant à eux, les protéines de surface jouent un large éventail de fonctions; adhésion à plusieurs ligands, résistance au complément et aux anticorps, invasion des cellules de l'hôte, etc[28].

Ces composants exprimés en surface représentent des facteurs de virulence potentiels, ou confirmés, et une meilleure compréhension fondamentale de comment ces composants affectent la pathogenèse de l'infection et la virulence de *S. suis* demeure un enjeu de santé publique et économique.

Le but de cette thèse était de mieux caractériser l'impact de ces facteurs dans la pathogenèse de l'infection causée par *S. suis*. Cette thèse s'intéressait particulièrement à l'impact de la structure et de la composition de la CPS sialylée sur la virulence de *S. suis*. De plus, nous avons étudié le rôle de certaines protéines de surface non caractérisées à ce jour, soit Sao et les protéines liant le facteur H, dans la pathogenèse de *S. suis.*

La discussion des résultats présentés dans cette thèse se divise en trois grandes sections;

1- Rôle de la structure et de la composition de la CPS de *S. suis* sur ces propriétés fonctionnelles

2- Rôle de l'acide sialique sur la structure et la synthèse de la CPS chez *S. suis,* en comparaison avec GBS

3- Rôle des protéines de surface ancrées à la paroi, Sao et des protéines liant le facteur H, dans la pathogenèse de l'infection causée par *S. suis*

1. Rôle de la structure et de la composition sur les propriétés fonctionnelles de la CPS de *S. suis*

1.1. Caractérisation de nouveaux loci capsulaires (NLCs) chez des souches de *S. suis* non-sérotypables

Il faut remonter en 1966 pour voir la reclassification des sous-groupes S, R, et RS appartenant au groupe D de Lancefield en une nouvelle espèce; *Streptococcus suis*. Les sous-groupes S, R et RS furent alors rebaptisés en sérotypes capsulaires 1, 2 et 1/2, respectivement. En vingt ans, entre les années 1983 et 2013, 32 nouveaux sérotypes ont été décrits également basés sur l'antigénicité de la CPS, portant le nombre à 35 différents sérotypes (1/2 + 1-34). Récemment, avec l'accessibilité des technologies de séquençage, les traits génétiques de l'ensemble des sérotypes ont pu être comparés. Ainsi, comme mentionné dans la section 'Revue de littérature', certains sérotypes ont été reclassifiés en d'autres espèces que *S. suis*, diminuant le nombre de 'vrais' sérotypes à 29[13]. Cependant, la disponibilité des technologies de séquençage et des banques de données ont également contribué à la découverte de 17 nouveaux loci capsulaires chez des souches non-sérotypables. Or, ces souches ont toutes été isolées de Chine et, pour la majorité, de porcs sains. Ces souches pourraient représenter de nouveaux types capsulaires endémiques à la Chine[15, 214, 215].

Au moment d'amorcer cette thèse, très peu d'informations étaient disponibles concernant les souches non-sérotypables isolées du Québec. Comme démontré en annexe dans l'**article VII**, dont je suis coauteur, les souches non-sérotypables représentent un pourcentage non négligeable des souches isolées de porcs malades au Québec entre les années 2008 et 2011. Les souches non-sérotypables isolées dans ces années représentent entre 14.5% et 33.2% du total des souches caractérisées. Un nombre considérable des souches non-sérotypables ont été isolées de différents organes, notamment dans les méninges, suggérant que ces souches peuvent être virulentes et capables de coloniser les organes et les tissus. Par exemple, entre 2008 et 2011, 18% des souches isolées des méninges sont non-sérotypables, alors que 16.5% d'entre elles correspondent au sérotype 2. De plus, bien que 89% des souches démontrent une grande hydrophobicité et sont probablement peu ou pas encapsulées, 11% de

ces souches sont potentiellement encapsulées. Ces résultats suggèrent que ces souches non-sérotypables pourraient représenter de nouveaux types capsulaires avec une représentation épidémiologique considérable au Québec.

Étant donné que ces nouveaux types capsulaires peuvent posséder, en théorie, une structure, une composition et un locus capsulaire différents des autres sérotypes connus, nous avons génétiquement caractérisé les loci capsulaires de plusieurs souches non-sérotypables isolés au Québec (Canada) (**ARTICLE I**).

Selon les observations obtenues avec les souches non-sérotypables et les NCLs, trois causes peuvent expliquer le phénotype non-typable de ces souches; (i) souches de sérotypes connus, mais non-encapsulées, (ii) souches dépourvues totalement de locus capsulaire et (iii) souches appartenant à des NCLs (caractérisés ou non) [15, 214, 215].

Le typage moléculaire par PCR multiplexe des 79 souches de *S. suis* non-sérotypables isolés de porcs malades au Québec a révélé la présence de 15 souches (18.9%) de sérotypes connus, suggérant l'absence de production de CPS à la surface de ces 15 souches (i). L'analyse génétique des loci a permis de confirmer que des délétions, des insertions et des mutations ponctuelles, probablement responsables de l'inhibition de la synthèse de la CPS. La présence de mutation ne semble pas résulter automatiquement en un phénotype non-encapsulé irréversible puisqu'il a été démontré que certaines souches mutées retrouvent leur phénotype encapsulé après un passage *in vivo*[269].

Une observation intéressante est la présence de 47 souches appartenant à des NCLs déjà caractérisés chez des souches isolées des porcs sains majoritairement (iii). De plus, 14 des 17 NCLs caractérisés ont été retrouvés chez les souches non-sérotypables du Québec; les NCLs 8, 9 et 15 n'ayant pas été retrouvés. La présence de presque tous les NCLs existants chez les souches isolées non-sérotypables au Québec suggère une grande distribution de ces NCLs à travers la population de *S. suis*. Les NCLs prédominant au Québec (3, 4, 7 et 17) sont néanmoins différents de ceux des souches isolées en Chine (1, 2, 3 et 7) et suggèrent un lien

potentiel entre la virulence et les NCLs. Curieusement, sur les 17 souches non-sérotypables restantes, 7 d'entre elles ne possèdent pas de locus capsulaire (ii).

Un résultat majeur de ces travaux est la découverte de 4 nouveaux NCLs, nommés NCL17-20, sur les dix souches non-sérotypables restantes (**Article I; Figure 1**). Les loci capsulaires de ces nouveaux NCLs sont très différents l'un de l'autre (**voir Tableau SII, Article I**), et comme tous les NCLs, les NCLs 17-20 codent chacun pour une polymérase (*wzy*) spécifique. Un fait intéressant est qu'aucun des NCLs 17-20 ne possède des gènes impliqués dans la synthèse de l'acide sialique. Il est important également de souligner que plusieurs gènes au sein des loci NCLs 17-20 sont associés à des HGs spécifiques à ces loci. On retrouve parmi ces gènes, la polymérase, la flippase et des glycosyltransférases. À titre de rappel, les HGs représentent des groupes de gènes codant pour des fonctions homologues. Ces résultats suggèrent que ces loci codent pour des gènes de synthèse de CPSs de compositions et de structures significativement différentes des autres sérotypes.

Pour résumé cette section sur la caractérisation des souches non-sérotypables du Québec, nous avons déterminé qu'une petite proportion des souches non-sérotypables isolées chez les porcs malades provenant de différentes fermes au Québec correspondaient à des sérotypes connus, mais l'absence ou la faible production de la CPS rend impossible le typage sérologique. La majorité des souches non-sérotypables isolées au Québec (72%) correspondent bien à des nouveaux NCLs. Bien que les études sur les souches non-sérotypables soient limitées pour l'instant à des isolats de la Chine et du Canada, certains NCLs caractérisés (NCLs 17-20) sont uniques aux souches isolées du Québec. Néanmoins, 89% des souches non-sérotypables du Québec isolées entre 2008 et 2011 sont peu ou pas encapsulées. Il est donc probable que plusieurs souches associées aux nouveaux NCLs isolées du Québec soient peu ou pas encapsulées. Une perspective intéressante sera de trouver des souches bien encapsulées de chaque NCLs afin de déterminer la structure de la CPS et puis d'obtenir des anticorps pour les tests diagnostiques sérologiques. Les nouvelles techniques de typage moléculaire, couplées à la détection de ces nouveaux NCLs, permettront de mieux caractériser l'épidémiologie de *S. suis*, considérant le pourcentage élevé que représente les souches non-sérotypables.

1.2 Propriété antiphagocytaire de la CPS de *S. suis* sérotype 14

Le rôle de la CPS du sérotype 2 dans la virulence de *S. suis* a été extensivement étudié dans les dernières années. Depuis l'année 2000, quarante-trois articles ont été publiés en lien avec la virulence et la CPS de *S. suis*. De ce nombre, vingt-huit (65%) ont été faits spécifiquement sur le sérotype 2[270] et très peu d'information sur les propriétés fonctionnelles de la CPS des autres sérotypes est disponible.

Comme décrit dans la section précédente, plusieurs autres structures capsulaires de *S. suis* pourraient exister et être prochainement décrites. Par contre, notre compréhension sur comment la structure de la CPS influence la virulence et la pathogenèse de l'infection causée par *S. suis* est très limitée. Comme décrit dans la section 'Revue de littérature', des études sur la fonctionnalité de la CPS avec différents sérotypes suggèrent un impact du type capsulaire sur certaines propriétés fonctionnelles, notamment dans la résistance à la phagocytose et dans la modulation de la réponse immunitaire de l'hôte. De plus, la prévalence de certains sérotypes dans les cas d'infections chez le porc et l'humain, notamment le sérotype 2, suggère un lien entre la virulence et le type capsulaire. Cependant, chez *S. suis,* aucune étude avec des mutants non-encapsulés, outre qu'avec le sérotype 2, n'avait pas été effectuée afin d'étudier les propriétés fonctionnelles de la CPS chez d'autres sérotypes.

De plus, il est proposé que le sérotype capsulaire influence également la pathogenèse de l'infection causée par plusieurs pathogènes. Chez GBS, par exemple, il a été démontré avec des mutants non-encapsulés que les CPSs du type III et du type V possèdent des propriétés légèrement différentes dans un modèle *in vitro* avec des cellules dendritiques porcines. En effet, les travaux auxquels j'ai participé (cités en annexe, **Article VII**), démontrent que la CPS du type III protège contre la destruction intracellulaire, alors que la CPS du type V ne semble pas avoir cette propriété. De plus, la CPS du type V semble protéger légèrement la bactérie de la phagocytose dans le temps, contrairement à la CPS du type III. Néanmoins, aucune différence majeure dans les patrons d'expressions de cytokines produites par les cellules

dendritiques n'a été observée, suggérant un effet mineur du type capsulaire entre GBS type III et V sur les interactions avec les cellules dendritiques.

Afin d'étudier les propriétés antiphagocytaires de la CPS de *S. suis* chez un type capsulaire différent du sérotype 2, nous avons construit des mutants de gènes du locus de la CPS chez *S. suis* sérotype 14 (**Article III**). À titre de rappel, les structures des CPSs des sérotypes 2 et 14 sont différentes (**Revue de littérature; Figures 4 et 5**). La CPS du sérotype 2 possède une chaîne latérale (Gal) supplémentaire reliée à un rhamnose, sucre d'ailleurs absent chez la CPS du sérotype 14. Deux mutants présentant un phénotype non-encapsulé ont été obtenus en mutant les gènes *cps14B* et *neu14C*[210, 212]. Le rôle de la protéine régulatrice CpsB a déjà été caractérisé chez *S. suis* sérotype 2 et il a été démontré que cette protéine était essentielle à la production de la CPS[49]. Considérant les fonctions conservées des gènes *cpsABCD* dans tous les sérotypes, il est normal d'obtenir également un phénotype non-encapsulé chez le mutant du gène *cps14B*. L'impact phénotypique de la délétion du gène *neu14C,* impliqué dans a synthèse de l'acide sialique chez le sérotype 14, sera discuté dans la section 2.

Les observations menées lors des tests de phagocytose avec des macrophages ont permis de déterminer que la CPS du sérotype 14 était, comme la CPS du sérotype 2, un important facteur antiphagocytaire, et ce, malgré les différences en composition et en structure entre les CPSs (**Article II, Figure 2**). Un résultat intéressant est la plus grande résistance à la phagocytose de la part de la souche mère du sérotype 14 par rapport à la souche mère du sérotype 2, suggérant un rôle de la structure dans les propriétés fonctionnelles de la CPS. Les images de microscopies électroniques (**Article III, Figure S1 et Article IV, Figure 4**), les rendements de production de CPS ainsi que la masse moléculaire des CPSs (**Article III, Table SII**) des CPSs des sérotypes 2 et 14 sont similaires et suggèrent une quantité comparable de CPS à la surface des souches de références des sérotypes 2 et 14, également utilisées dans cette étude. Cependant, la souche de sérotype 2 (ST1) et celle du sérotype 14 (ST6) représentent des patrons génétiques différents, tel qu'indiqué par le typage allélique (ST). De plus, les différences dans la résistance à la phagocytose observées entre le sérotype 2 et 14 n'ont pas pu être confirmées en présence de sérum (**Article III, Figure 7**). Par conséquent, les

différences observées dans la résistance à la phagocytose pourraient être également dues à d'autres facteurs.

Le rôle de la CPS du sérotype 14 dans la virulence de *S. suis* a également été évaluée dans un modèle *in vivo* murin. Il est bien connu que la CPS du sérotype 2 est un facteur de virulence critique lors d'infections expérimentales chez la souris, cependant aucune étude n'a évaluée l'impact de la CPS dans la virulence de *S. suis* sérotype 14[49]. Étant donné qu'aucun modèle d'infection expérimental murin pour le sérotype 14 n'a pas été décrit, nous avons adapté le modèle d'infection du sérotype 2 pour le sérotype 14. Les essais préliminaires avec des doses intrapéritonéales similaires utilisées chez le sérotype 2 (1-5 x 10^7 UFC) ont cependant donné qu'une très faible mortalité et très peu de signes cliniques chez des souris infectées avec la souche mère.

Nous avons établi pour le modèle d'infection expérimentale à *S. suis* sérotype 14 qu'une dose de 1.5 x 10^8 UFC était nécessaire pour obtenir des taux de mortalité acceptables, permettant de comparer la virulence entre la souche mère et un mutant. Lorsqu'on compare avec la dose utilisée chez le sérotype 2, une souche virulence du sérotype 14 semble beaucoup moins virulente que les souches virulentes du sérotype 2 dans un modèle murin. La souche utilisée pour le sérotype 14 est de type allélique ST6, un type allélique associé à une virulence intermédiaire. Or, il a été démontré chez le sérotype 2, que des souches ST25 et ST28 (STs associés à une faible virulence) étaient néanmoins capables d'induire un taux de mortalité élevé (47-73%) dans un modèle murin. La plus faible virulence observée chez la souche du sérotype 14 pourrait donc être en partie liée au sérotype (et donc potentiellement à la CPS), ou être une particularité de la souche testée.

Les résultats obtenus avec la souche mutante non-encapsulée du sérotype 14 ont permis de démontrer que la CPS du sérotype était un facteur de virulence critique. Non seulement les souris infectées avec la souche mutante n'ont présenté aucune mortalité, mais la bactérie a été très rapidement éliminée du sang comme le montre la bactériémie (**Article II, Figure 3**). À l'opposé, les souris infectées avec la souche mère encapsulée ont présenté une très grande mortalité et une bactériémie persistance. La différence dans la charge bactérienne

sanguine entre les souris infectées avec la souche mère ou le mutant, démontre également bien le rôle de la CPS dans la persistance et la dissémination de *S. suis*, et ce, indépendamment du sérotype.

Pour conclure cette section sur les propriétés de la CPS du sérotype 14, nous avons démontré que la CPS de *S. suis* sérotype 14 était un facteur antiphagocytaire critique pour la virulence de *S. suis*, malgré les différences de structure et de composition avec la CPS du sérotype 2. Les expériences *in vitro* et *in vivo* ont démontré le rôle majeur de la CPS du sérotype 14 dans la persistance et dans la dissémination de la bactérie. Des différences mineures dans les propriétés antiphagocytaires ont été observées dans un modèle *in vitro* entre le sérotype 2 et 14, cependant d'autres facteurs que le sérotype, notamment le patron génétique (expression de différents facteurs antiphagocytaires), pourraient expliquer ces différences. Curieusement, le sérotype 14 semble néanmoins moins virulent, malgré des propriétés antiphagocytaires comparables au sérotype 2, suggérant d'autres facteurs impliqués dans les différences de virulences.

1.3 Étude comparative des propriétés fonctionnelles des CPSs des sérotypes 1, 1/2, 2 et 14

Chez *S. suis*, la surreprésentation de certains sérotypes capsulaires et de STs dans le cas d'infections chez le porc et chez l'humain, suggère des rôles combinés de la CPS et du bagage génétique sur la virulence de *S. suis*[3]. Une limite importante dans l'utilisation de souches de différents sérotypes est le biais potentiel que représentent les variations génétiques entre les isolats. De plus, dans la plupart des études, les souches expriment des quantités différentes (voir inconnues) de CPS à leur surface et les propriétés fonctionnelles de celles-ci pourraient être corrélées avec l'épaisseur de la CPS.

Comme décrit dans la section 'Revue de littérature' (**Figures 4 et 5**), la structure de la CPS entre les sérotypes 1 et 14 et 1/2 et 2 sont identiques et la composition ne diffère que par un seul sucre. Ces sérotypes représentent donc des cibles intéressantes afin d'étudier l'impact de la modification d'un seul sucre sur les propriétés fonctionnelles de la CPS. De plus, il a

récemment été proposé qu'un seul polymorphisme nucléotidique (SNP) dans le gène *cpsK* (codant pour soit une galactosyltransférase ou une *N*-acétylgalactosyltransférase) serait potentiellement responsable de la différence de la composition de la CPS entre les sérotypes 1/2 et 2 et les sérotypes 1 et 14. La construction de mutants isogéniques est donc théoriquement possible en substituant le SNP à l'acide aminé 483 dans le gène *cpsK*.

Dans le but d'étudier l'impact du type capsulaire chez des mutants isogéniques, mais également de tester l'hypothèse qu'un seul SNP différencie les sérotypes 1/2 et 2 et les sérotypes 1 et 14, nous avons créé des mutants isogéniques de chacun de ces sérotypes. Nous avons muté le nucléotide 483 pour un T chez les sérotypes 1 et 1/2 (G483T) et pour un G chez les sérotypes 2 et 14 (T483G). Il est intéressant de mentionner également que les souches de références utilisées représentent trois types de patrons génétiques différents. Nous avons utilisé des souches appartenant à des STs associés à une haute virulence (ST1), une virulence intermédiaire (ST6) ainsi qu'une virulence faible (ST28) afin d'étudier l'impact du type allélique dans la virulence en fonction du type de CPS exprimé.

Des mutants isogéniques des sérotypes 1/2 et 2 exprimant une CPS d'un sérotype 2 et 1/2, respectivement, ont été obtenus en remplaçant l'acide aminé 161 du gène *cpsK*. Parallèlement, des mutants isogéniques des sérotypes 1 et 14 exprimant une CPS d'un sérotype 14 et 1, respectivement, ont également été obtenus en remplaçant le même acide aminé du gène *cpsK*.

Premièrement, les mutants obtenus nous ont permis de confirmer par tests sérologiques que la spécificité de l'acide aminé à la position 161 du gène *cpsK* influençait l'antigénicité de la CPS (**Article III; Figure 2**). Ces résultats confirment qu'un seul polymorphisme nucléotidique (nucléotide 483) dans le gène codant pour une glycosyltransférase (ici une chaîne latérale) est suffisant pour changer la spécificité du sucre transféré par la glycosyltransférase CpsK et ainsi modifier l'antigénicité de la CPS. Ceci est en accord avec les résultats de Van Calsteren & al. qui ont démontré que la chaîne latérale était un épitope capsulaire important[14]. Nos résultats démontrent bien que la modification du sucre galactose

sur la chaîne latérale des sérotypes 2 et 14 par un *N*-acétylgalactosamine chez les sérotypes 1/2 et 1 influence radicalement l'antigénicité de la CPS.

Chez les mutants des sérotypes 2 et 14, les analyses chimiques des CPSs purifiées ont permis de confirmer que la présence d'une cystéine à l'acide aminé 161 du gène *cpsK* (T161C) permet de catalyser le transfert d'un *N*-acétylgalactosamine à la place du galactose. À l'opposé, chez les mutants des sérotypes 1 et 1/2, la présence d'un tryptophane à l'acide aminé 161 du gène *cpsK* (C161T) permet de catalyser le transfert du galactose à la place d'un *N*-acétylgalactosamine[213]. La glycosyltransférase CpsK comporte deux domaines séparés par une région qui lie l'accepteur saccharidique. Curieusement, le polymorphisme nucléotidique se trouve dans le domaine fonctionnel de la glycosyltransférase. Les analyses structurelles de CpsK en 3D ont permis de confirmer que le résidu 161 influençait bien la spécificité du sucre saccharidique transféré (**Article III; Figure 5**). Il a été démontré que C161 dans Cps1/2K et Cps1K peut stabiliser le résidu GalNAc dans le substrat UDP-GalNAc en créant un lien polaire avec le groupe acétyle. À l'opposé, l'acide aminé W161 présent chez Cps2K et Cps14K permet d'accommoder un substrat plus petit, le galactose. De plus, chaque forme de CpsK peut uniquement stabiliser son ligand naturel (UDP-Gal ou UDP-GalNAc) et la substitution entre Gal et GalNAc (ou vice versa) n'est pas possible.

Une observation phénotypique importante chez les mutants isogéniques obtenus est la présence d'une encapsulation similaire aux souches mères. Il est connu que la modification du locus capsulaire peut influencer l'efficacité de polymérisation de la CPS. Chez GBS type III, il a été démontré que l'absence d'un sucre, soit l'acide sialique, influençait quantitativement la polymérisation, et probablement l'épaisseur de la CPS. Dans cette étude, les mutants isogéniques obtenus des sérotypes 1, 1/2, 2 et 14 démontrent tous un phénotype encapsulé comparable à leur souche mère respective.

De plus, il a été démontré que la longueur des chaînes polysaccharidiques était conservée chez les mutants exprimant une capsule de sérotype 2 et 14 (**Article III; Tableau supplémentaire S2**). Ces résultats suggèrent que la modification d'un galactose par un *N*-acétylgalactosamine, et vice versa, n'influence pas l'efficacité de la polymérase (quantité de CPS polymérisée) et

n'influence pas la régulation de la longueur des chaînes polysacccharidiques par le système codé par les gènes *cpsABCD*. Néanmoins, les deux mutants exprimant une capsule de sérotype 1 et 1/2 démontrent des longueurs de chaînes réduites lorsque comparés aux souches mères de sérotype 1 et 1/2, respectivement. Cependant, des expériences supplémentaires sont nécessaires pour confirmer ces différences observées.

Ensuite, pour la première fois chez *S. suis*, nous avons évalué l'impact du sérotype capsulaire dans la résistance à la phagocytose et dans la virulence avec les mutants isogéniques exprimant des CPSs de sérotypes différents. Curieusement, aucune différence dans les propriétés antiphagocytaires n'a été observée entre les souches mères et leur mutant exprimant une CPS modifiée (**Article III; Figure 7**). La modification d'un seul sucre ne semble donc pas influencer les propriétés antiphagocytaires de la CPS entres les sérotypes 1/2 et 2 et entres les sérotypes 1 et 14. D'un autre côté, outre la composition différence, la structure des chaînes polysaccharidiques de la CPS demeure identique. Une hypothèse est que les propriétés fonctionnelles de la CPS soient reliées à la structure des chaînes polysaccharidiques et non à la composition, comme hypothétisé à la section précédente chez les sérotypes 2 et 14.

De plus, nous avons démontré dans un modèle d'infection expérimentale murin que la virulence de *S. suis* est médiée davantage par le bagage génétique que par le type capsulaire (**Article III; Figure 6**). En effet, aucune modulation de la virulence n'a été observée chez une souche virulente ST1 exprimant une CPS provenant d'une souche de faible virulence (ST28), et vice versa. Parallèlement, aucune différence de virulence n'a été observée entre les mutants isogéniques exprimant une CPS de sérotype 14 et 1 et leurs souches mères de sérotypes 1 (ST1) et 14 (ST6), respectivement.

Ces observations sont en contraste avec celles effectuées chez *S. pneumoniae*. En effet, chez *S. pneumoniae*, la modification du type capsulaire influence plusieurs propriétés fonctionnelles de la CPS dont la déposition du complément et la résistance à l'opsonophagocytose. Néanmoins, chez *S. pneumoniae*, les mutants isogéniques ont été obtenus en remplaçant le locus capsulaire en entier et les mutants résultants expriment des CPSs significativement différentes (structures et compositions)[242, 245]. Chez *S. suis*, les CPSs

des mutants utilisés diffèrent que par un seul sucre et les résultats suggèrent que les impacts biologiques pourraient être minimes malgré l'antigénicité différente. Malgré que le changement de CPS ne semble pas avoir de répercussions sur la virulence et la résistance à la phagocytose chez les sérotypes testés, il serait néanmoins intéressant d'étudier *in vitro* certaines autres propriétés fonctionnelles de la CPS à partir de ces mutants, comme la déposition du complément et le recrutement du facteur H à la surface de la bactérie.

Pour conclure, c'est la première fois que des mutants isogéniques exprimant une CPS d'un sérotype différent sont obtenus chez *S. suis* et représentent, par conséquent, des outils uniques pour étudier l'impact du type capsulaire sur la virulence. De plus, ces travaux mettent en lumière les causes des réactions croisées observées entre les sérotypes 1/2 et 2 et les sérotypes 1 et 14. La différence d'antigénicité médiée par un seul SNP pourrait indiquer la présence d'un ancêtre commun pour les sérotypes 1/2 et 2 et pour les sérotypes 1 et 14. La mutation spontanée de l'acide aminé 121 du gène *cpsK* pourrait expliquer l'émergence de ces différents sérotypes capsulaires qui auraient ensuite été conservés dans la population de *S. suis*. Au final, ces travaux démontrent que les CPSs des sérotypes 1/2 et 2 et des sérotypes 1 et 14 possèdent des propriétés fonctionnelles similaires, malgré une composition et une antigénicité différente. La détermination des structures capsulaires des autres sérotypes couplés avec la disponibilité de banques de données sur les séquences des locus capsulaires permettra sans doute de caractériser d'autres polymorphismes responsables de réactions croisées entre sérotypes, notamment entre les sérotypes 6 et 16.

2- Rôle de l'acide sialique sur la structure et la synthèse de la CPS chez *S. suis,* en comparaison avec le streptocoque du Groupe B

Comme décrit dans la section 3 (Acide sialique) de la revue de littérature, l'acide sialique est incorporé dans les composants de surface de plusieurs pathogènes. La sialylation du LPS et de la CPS semble protéger les bactéries contre la déposition du complément, l'opsonophagocytose et module l'activation de certains types de Siglecs[248]. La majorité de nos connaissances sur la sialylation des composants de surface sont basées sur la synthèse de la CPS polysialylée d' *E. coli* et de *N. meningitidis*. À l'opposé, très peu d'études ont caractérisé le rôle de l'acide sialique dans la synthèse de la CPS chez les bactéries à Gram positif. *S. suis* et GBS sont les seules bactéries à Gram positif à incorporer de l'acide sialique dans leur composant de surface, soit plus précisément dans leur CPS. Les prochaines sections discuteront de l'impact de l'acide sialique dans la synthèse et la structure de la CPS chez *S. suis,* en comparaison avec GBS.

2.1 Rôle de l'acide sialique sur la synthèse de la CPS chez *S. suis,* en comparaison avec GBS

2.1.1 Chez *S. suis*

Premièrement, dans le but de déterminer si le locus de la CPS est régulé par un seul promoteur comme chez GBS, nous avons utilisé la technique de transcription inverse pour déterminer si le transcrit du locus capsulaire était polycistronique[232]. Il a été démontré qu'un seul promoteur était présent en amont du locus capsulaire et que tous les gènes, y compris ceux codant pour la synthèse de l'acide sialique, sont régulés à partir du même promoteur.

Nous avons ensuite étudié le rôle de l'acide sialique dans la synthèse de la CPS chez *S. suis*. Il a déjà été démontré que la disponibilité de l'acide sialique était critique pour la synthèse de la CPS chez le sérotype 2 (Δ*neu2C*)[217] et le sérotype 14 (Δ*neu14C*; Article II). Or, aucun mutant de la sialyltransférase n'a jamais été obtenu étant donné la létalité de la mutation.

Dans le but de contourner la létalité de cette mutation, nous avons développé une approche de mutagenèse en trois étapes (voir méthodologies et résultats).

Les observations phénotypiques ont démontré que la réintroduction du gène *neu2C* n'est pas suffisante pour lever l'inhibition de la production de la CPS. De plus, la présence de d'autres mutations ponctuelles suppressives dans le locus peut être exclue, considérant la restauration partielle de la CPS sialylée chez le mutant complémenté. Ces résultats confirment que l'acide sialique est nécessaire à la synthèse de la CPS chez *S. suis*, et que les phénotypes observés sont probablement spécifiques à l'espèce. Étant donné que nous avions également démontré le rôle critique de l'acide sialique chez le sérotype 14, nous n'avons pas fait de mutant équivalent (*cps14N*) chez ce sérotype étant donné le phénotype non-encapsulé chez le sérotype 2.

L'absence d'acide sialique sur les chaînes polysaccharidiques de *S. suis* pourrait empêcher la reconnaissance des sous-unités par la flippase ou par la polymérase afin d'exporter ou de polymériser la CPS, respectivement. Par contre, ceci devrait en théorie causer également une séquestration du précurseur lipidique. Alternativement, le complexe de régulation formé par CpsB et CpsC est également responsable de la translocation des chaînes polysaccharidiques dans le peptidoglycane[220]. L'absence de chaîne complète (sialylée) pourrait également empêcher ce complexe de transférer ou ancrer efficacement les chaînes polysaccharidiques dans le peptidoglycane, mais aussi de recycler le précurseur, d'où l'absence de CPS à la surface. Les causes exactes de la différence observée entre l'absence de la disponibilité de l'acide sialique (phénotype non-encapsulé) et l'absence de l'enzyme sialyltransférase (létale) chez *S. suis* restent à être élucidé.

2.1.2 Chez GBS

Chez GBS type III, il a été démontré que la CPS peut être synthétisé en l'absence de la synthèse de l'acide sialique (*neu3B* et *neu3D*[217, 238]). Étonnamment, chez GBS type III, la mutation dans la sialyltransférase n'est pas létale et résulte en un phénotype encapsulé diminué, mais dépourvu d'acide sialique[232]. Chez GBS, le rôle de l'acide sialique dans la

synthèse de la CPS ce restreint au type III et ce, malgré la présence de l'acide sialique dans tous les types capsulaires de GBS. Afin de comparer le rôle de l'acide sialique dans la synthèse de la CPS chez d'autre type capsulaire de GBS, nous avons créé des mutants déficients dans la synthèse de l'acide sialique (*neu5B*) chez GBS type V. De plus, nous avons évalué la létalité potentielle d'une mutation dans le gène de la sialyltransférase *cps5K* chez GBS type V. Les phénotypes encapsulés dépourvus d'acide sialique obtenus chez les mutants Δ*neu5B* et Δ*cps5K* confirment que la CPS de GBS type V peut également être synthétisé en absence d'acide sialique et que ce sucre est nécessaire pour la production optimale de la CPS (**Article IV; Figure 11**). La mutation dans le gène de la sialyltransférase (*cps5K*) est également non-létale chez GBS type V, contrairement à *S. suis*. La raison de cette différence est encore inconnue, mais une raison probable pourrait être liée à la spécificité des enzymes clés de la voie de synthèse de la CPS; la polymérase et la flippase. Chez GBS, une moins grande spécificité de la polymérase et/ou de la flippase pour les sous-unités sialylées permettrait d'exporter ou de polymériser assez de CPS pour recycler le précurseur lipidique pour son utilisation dans les autres voies biologiques de la bactérie (ex.: synthèse du peptidoglycane). D'ailleurs, il a été démontré que la synthèse de la CPS chez GBS est réduite significativement en absence d'acide sialique.

Une différence intéressante entre la production de la CPS du type III et type V en absence d'acide sialique est la longueur des chaînes polysaccharidiques. Chez GBS type III, il a été démontré que les mutants asialylés produisaient des chaînes polysaccharidiques pour longues que la souche mère[232, 238]. À l'opposé, chez le type V, nous avons remarqué chez les deux mutants asialylés (Δ*neu5B* et Δ*cps5K*) une production des chaînes polysaccharidiques plus courtes que leur souche mère. La longueur des chaînes est contrôlée par le système de phosphorégulation codé par les gènes *cpsABCD* ce qui suggère une différence dans ce système de régulation entre les types III et V. Une autre hypothèse serait la moins grande spécificité de la polymérase du type V pour les sous-unités sans acide sialique ce qui influencerait négativement la polymérisation, comparativement au type III (**Section 2.5**). Chez le type V, la diminution de la quantité de CPS exprimée à la surface est probablement due à la diminution de la quantité de sous-unités transférées en surface, et à une diminution de la longueur des chaines polysaccharidiques.

2.2. Rôle de la spécificité du lien glycosylique de l'acide sialique sur la structure et synthèse de la CPS de *S. suis*, en comparaison avec GBS

Curieusement, on retrouve une variation dans la nature du lien glycosylique entre l'acide sialique et le galactose porté dans la chaîne latérale entre *S. suis* (α2,6) et GBS (α2,3). Il est proposé que cette différence dans le lien de l'acide sialique pourrait influencer les propriétés immunomodulatrices de la CPS sialylée, mais également la synthèse de la CPS. Dans le but d'étudier l'impact du lien glycosylique de l'acide sialique, nous avons créé une approche de mutagenèse dans le but de substituer le gène codant pour la sialyltransférase (α2,6 ou α2,3) entre *S. suis* sérotypes 2 et 14, et GBS types III et V.

2.2.1. α2,3-sialyltransferase chez *S. suis* sérotypes 2 et 14

Étant donné que plusieurs essais de substitution directe du gène de la sialyltransférase ont été tentés sans succès chez *S. suis*, probablement dû à la létalité de la mutation[220], nous avons utilisé la même approche que celle utilisée pour muter le gène de la sialyltransférase chez le sérotype 2 (Δ*cps2N*). Nous avons ainsi obtenu deux mutants, chez les sérotypes 2 et 14, possédant le gène de sialyltransférase de GBS type III. Malheureusement, les mutants obtenus démontrent des phénotypes non-encapsulés (**Article IV; Figures 4 et 5**). Ces résultats confirment l'impossibilité d'obtenir des mutants de *S. suis* exprimant une CPS sans acide sialique, ou avec un lien modifié de l'acide sialique. Il a été proposé que les chaînes polysaccharidiques de la CPS de *S. suis* doivent être sialylées en α2,6 pour être exportées et polymérisées. Chez *S. suis*, les sialyltransférases des sérotypes 2 et 14 sont identiques à 100%, suggérant qu'elles reconnaissent un épitope précis partagé chez ces deux structures. Or, les sialyltransférases de *S. suis* et de GBS type III démontrent seulement 33% d'identité protéique. La sialyltransférase de GBS type III pourrait donc être incapable de sialyler les chaînes polysaccharidiques de *S. suis*. À l'opposé de *S. suis*, les sialyltransférases de GBS varient entre les types capsulaires et suggèrent qu'elles reconnaissent des motifs uniques à chaque structure[231]. Ainsi, on ne peut pas exclure que la sialyltransférase de GBS type V serait capable de sialyler les chaînes polysaccharidiques de *S. suis*.

2.2.2. α2,6-sialyltransférase chez GBS types III et V

Nous avons démontré précédemment que la synthèse de la CPS chez GBS semble plus permissive que chez *S. suis*. Afin de déterminer si GBS peut exprimer une CPS présentant un lien modifié de l'acide sialique, nous avons substitué le gène codant pour la sialyltransférase de GBS types III et V (α2,3) par le gène codant pour la sialyltransférase de *S. suis* (α2,6).

Les deux mutants obtenus chez les types III et V expriment une CPS modifiée dans le lien de l'acide sialique (α2,6 au lieu de α2,3). Curieusement, tout comme les mutants de la voie de synthèse de l'acide sialique (*neu5B*) et de la sialyltransférase (*cpsK*), les mutants avec une α2,6-sialyltransférase expriment une épaisseur de CPS diminuée (**Article IV; Figures 10 et**

11). Une autre similarité avec les mutants Δ*neu5B* et Δ*cps5K*, est la longueur diminuée des chaînes polysaccharidiques. Il semble que le lien glycosylique de l'acide sialique influence l'exportation, la polymérisation et/ou la translocation des chaînes polysaccharidiques chez GBS.

Dans un travail en collaboration, la CPS purifiée du mutant de GBS type III sialylée en α2,6 a été testée pour étudier la possible différence d'immunogénicité entre les CPSs sialylées en α2,3 et α2,6. Ces travaux font d'ailleurs partis d'un article scientifique publié dont je suis co-auteur (**Annexe; Article IX**). Il a été démontré que la modification du lien de l'acide sialique (α2,3 en α2,6) résulte en la perte complète de la reconnaissance de la CPS par les anticorps dirigés contre la CPS native (α2,3), suggérant un effet important de la spécificité du lien de l'acide sialique α2,3 dans l'antigénicité (épitope) de la CPS de GBS type III. Il est cependant important également de mentionner qu'une faible réponse anticorps a été rapportée contre la CPS modifiée en α2,6, suggérant la génération d'un nouvel épitope immunologique.

Pour conclure cette section, il a été démontré que la synthèse de la CPS chez *S. suis* est stricte et que la présence d'acide sialique α2,6 est crucial à la synthèse de la CPS à la surface. On pourrait caractériser cette particularité propre à *S. suis* comme un 'tout ou rien'. *S. suis* pourrait avoir recours à cette stratégie afin de conserver la sialylation de sa capsule au sein de sa population, suggérant un rôle important de ce sucre chez *S. suis*. À l'opposé, malgré que la synthèse de la CPS semble plus permissive chez GBS, les résultats de ces travaux démontrent qu'il existe quand même des différences au sein de l'espèce. L'absence d'acide sialique influence différemment la polymérisation des chaînes polysaccharidiques entre GBS types III et V. Cependant, il a été démontré que l'acide sialique en α2,3 était nécessaire, mais non crucial, à la production de la CPS chez GBS, et ce chez les deux types capsulaires testés (III et V). De manière plus importante, la α2,6-sialyltransférase de *S. suis* est capable de sialyler les sous-unités polysaccharidiques de GBS types III et V. Ces mutants exprimant un lien de l'acide sialique modifié représentent des outils intéressants pour étudier l'impact du lien de l'acide sialique dans la virulence de ces pathogènes. Cependant, d'autres travaux supplémentaires sont nécessaires afin de comprendre en détail le procédé d'assemblage et de régulation de la synthèse de la CPS chez *S. suis* et GBS.

3. Rôle des protéines de surface ancrées à la paroi, Sao, Fhbp et Fhb, dans la pathogenèse de l'infection causée par *S. suis.*

3.1. Rôle de la protéine de surface Sao dans la pathogenèse de l'infection causée par *S. suis*

Les recherches pour trouver des candidats immunogéniques pour le développement de vaccins sous-unitaires ont mené à la découverte de différentes protéines immunogéniques chez *S. suis*; dont Sao, MRP, EF, suilysin, etc[59]. En général, les antigènes peuvent être regroupés en différentes classes selon leurs fonctions et ligands, et plusieurs de ces classes d'antigènes sont impliquées dans la virulence des pathogènes. Une classe d'antigène bien connu chez les bactéries à Gram positif est celle comprenant les antigènes I/II qui contribuent à l'adhérence à la matrice extracellulaire. J'ai d'ailleurs contribué aux travaux qui ont permis de caractériser le rôle de ces antigènes I/II dans la virulence de *S. suis* (**Annexe, Article XI**).

Néanmoins, le rôle exact de certaines protéines dans la pathogenèse causée par *S. suis* demeure inconnu. C'est le cas de la protéine immunogénique « Surface Antigen One » ou Sao exprimée à la surface de *S. suis*. À l'aide d'un mutant isogénique déficient dans la production de Sao, nous avons évalué le rôle de cette protéine dans la pathogenèse de l'infection causée par *S. suis*. Étonnamment, aucune différence dans l'invasion, l'adhésion, la résistance à la phagocytose et dans la virulence de *S. suis* n'a été observé entre le mutant et la souche mère (**Article VI**; Figures 2 et 3). Ces résultats sont surprenants, car plusieurs antigènes exprimés chez les bactéries à Gram positif sont impliqués dans l'adhésion. Il est cependant possible que Sao lie des ligands présents sur d'autres types de cellules, notamment chez les cellules endothéliales ou des ligands espèce-spécifiques. Néanmoins, la virulence du mutant Δ*sao* n'est pas atténuée dans un modèle murin et, par conséquent, n'est pas un facteur de virulence critique dans le modèle testé.

Malgré le caractère immunogénique de Sao, son rôle dans la pathogenèse de l'infection causée par *S. suis* semble limité. Nous avons démontré pour la première fois que Sao a un rôle négligeable dans la colonisation, dans la dissémination et dans la virulence de *S. suis*. Sao est néanmoins une cible intéressante pour le développement d'un vaccin sous-unitaire étant donné

sa capacité à indure d'anticorps opsonisants dirigés contre Sao chez le porc. Finalement, comme le démontre un article dont j'ai participé comme coauteur (**Annexe; Article XII**), le rôle attribué à certains facteurs peut varier selon le patron génétique de la souche utilisée ou encore du modèle expérimental utilisé, conséquemment, il n'est pas exclut que Sao puisse jouer un rôle dans la virulence de souches génétiquement différentes.

3.2. Rôle des protéines Fhbp et Fhb liant le facteur H dans la pathogenèse de l'infection causée par *S. suis*

La colonisation des cellules épithéliales des voies respiratoires supérieures est une première étape cruciale lors de l'infection causée par *S. suis*[3, 28]. La majorité des interactions entre les bactéries et les cellules de l'hôte sont médiées par un large éventail d'adhésines exprimées à la surface de la bactérie. Les adhésines présentes à la surface de *S. suis* sont typiquement des macromolécules qui interagissent avec des hydrates de carbone spécifiques ou des protéines (récepteurs) présentes à la surface des cellules cibles[28]. L'adhérence de la bactérie à certaines surfaces peut être médiée par des liaisons réversibles non spécifiques, par exemple via les forces électrostatiques et des liens hydrophobiques. L'adhésion peut également être ligand-spécifique. Un bon exemple est la protéine Fbps de *S. suis* qui est capable de lier la fibronectine[96].

Le facteur H, quant à lui peut être considéré comme un médiateur d'adhérence. Il a été démontré que la protéine liant le facteur H PspC chez *S. pneumoniae* contribue au recrutement du facteur H et favorise l'adhérence aux cellules épithéliales et endothéliales[87]. De plus, outre son rôle décrit dans l'adhérence, le recrutement du facteur H par les composants de surface pourrait protéger contre l'activation du complément et contre l'opsonophagocytose[91]. À titre de rappel, deux protéines différentes ont été caractérisées chez *S. suis* pour leurs capacités à lier le facteur H humain, Fhb et Fhbp. Il a été démontré que la protéine Fhb contribuait à la résistance à la phagocytose et à la virulence dans un modèle d'infection expérimentale porcin[77]. Pour sa part, aucune étude n'a caractérisé le rôle de Fhbp dans la pathogenèse de l'infection causée par *S. suis*[78].

Nous avons tout d'abord évalué le rôle du facteur H dans l'adhérence de *S. suis* aux cellules épithéliales pulmonaires humaines (A549) et aux cellules endothéliales microvasculaires du cerveau humaine (hBMEC). Nous avons décidé de travailler avec un modèle de cellules humaines étant donné que les protéines Fhb et Fhbp ont été caractérisées selon leurs capacités à lier le facteur H humain. Au moment d'écrire cette thèse, aucune étude n'avait adressé l'interaction des protéines Fhb et Fhbp avec le facteur H porcin, ou d'une autre espèce (murin). Chez d'autres pathogènes, notamment chez *N. meningitidis* et *Neiserria gonorrheae,* il a été démontré que le recrutement du facteur H est spécifique au facteur H humain[85, 271]. Chez *N. gonorrheae* en particulier, il a été démontré que le recrutement du facteur H est spécifique au facteur H humain et que ce pathogène a une faible affinité pour le facteur H de d'autres primates. Le recrutement du facteur chez *S. suis* pourrait être également espèce-spécifique.

Les expériences préliminaires avec la souche de *S. suis* sérotype 2 P1/7 ont démontré que la présence de facteur H à la surface de *S. suis* agit également comme médiateur de l'adhérence de *S. suis* aux cellules épithéliales et endothéliales (**Article V; Figure 2**). Dans le but de caractériser le rôle des protéines liant de facteur H, Fhb et Fhbp, dans la pathogenèse de *S. suis*, nous avons construit des mutants simples et doubles de ces protéines. Nous avons ensuite évalué ces mutants, simples et doubles, dans l'adhésion et l'invasion en présence et absence de facteur H humain. En absence de facteur H, aucune différence significative dans l'adhérence et dans l'invasion n'a été observée par rapport à la souche mère, et ce, chez les deux types cellulaires testés, suggérant un rôle limité de ces protéines dans l'adhésion spécifique aux cellules. Par contre, en présence de facteur H humain, nous avons observé une diminution significative de l'adhésion aux cellules épithéliales et endothéliales du double mutant déficient dans la production des protéines Fhb/Fhbp (**Article V; Figure 3**). Les différences observées uniquement chez le double mutant suggèrent une compensation dans la liaison au facteur H par l'autre protéine chez les simples mutants de *fhb* et *fhbp*. Curieusement, les quantités de facteur H à la surface des différents mutants sont toutes comparables à la souche mère. Comme mentionné dans la section 'Revue de littérature', d'autres composants de surface ont été caractérisés pour leur capacité à recruter le facteur H. On y retrouve notamment

les composants de surface sialylés (LPS et CPS). Afin d'évaluer le potentiel de la CPS sialylée à recruter le facteur H, nous avons construit des mutants non encapsulés chez la souche mère, mais également chez le double mutant *Δfhb/Δfhbp.* Ces mutants nous ont permis de démontrer qu'effectivement, la CPS était capable de recruter le facteur H (**Article V; Figure 5**).

Le facteur H contient 20 différents domaines. Les pathogènes sont capables de lier le facteur H à différents domaines, résultant en une orientation distincte à la surface de la bactérie (**Revue de littérature, Figure 2**)[79]. Dépendamment des domaines présentés à la surface de la bactérie, le facteur H exercera donc des fonctions différentes (résistance au complément ou adhésion) (**Figure 1**). Le niveau d'adhésion en présence de facteur H n'est donc pas relié directement à la quantité de facteur H à la surface de la bactérie. Une observation intéressante est la présence d'une petite quantité de facteur H chez le triple mutant *Δfhb/Δfhbp/Δcps,* suggérant la présence de d'autres facteurs permettant le recrutement du facteur H. À la suite de la publication de notre article présentant ces résultats, une autre étude portant sur le recrutement du facteur H par *S. suis* a rapporté la présence de 14 autres protéines potentiellement capables (huit confirmées) de lier (spécifiquement ou non) le facteur H[272].

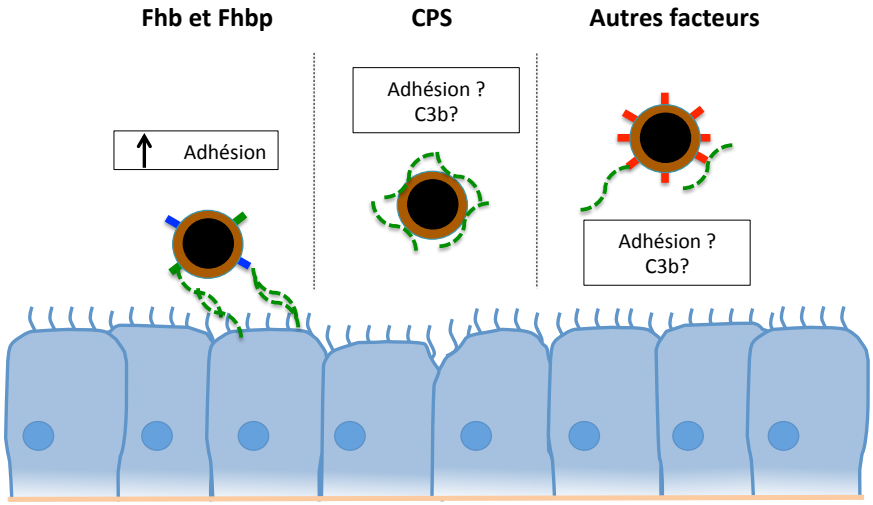

Figure 1. Recrutement et orientation du facteur H en fonction du récepteur au facteur H utilisé à la surface de *S. suis*. Nous avons démontré que les protéines Fhb et Fhbp contribuent à l'adhésion de

S. suis par le recrutement du facteur H par une ou plusieurs régions du facteur H encore inconnues. Le rôle biologique du recrutement du facteur H par la CPS de *S. suis* sérotype 2 demeure à être caractérisé. Finalement, d'autres protéines peuvent également recruter le facteur H, mais leur impact sur la pathogenèse de l'infection causée par *S. suis* demeure hypothétique.

Nous avons ensuite évalué le rôle du facteur H recruté en surface dans la dégradation du complément (C3b) et dans la résistance à la phagocytose. Curieusement, il a été observé que le triple mutant Δ*fhb*/Δ*fhbp*/Δ*cps* possède toujours la capacité de recruter le facteur H comme cofacteur afin de dégrader le C3b en sa forme inactive (**Article V; Figure 6**). Ce résultat confirme la présence d'au moins un autre facteur additionnel à la surface de *S. suis* impliqué dans le recrutement du facteur H et la dégradation du complément.

Malgré la capacité du facteur H recruté en surface à dégrader le complément, aucune différence dans la résistance à la phagocytose en présence de sérum humain n'a été observée chez les mutants simples et doubles des protéines Fhb et Fhbp. Ces conclusions sont différentes de celles rapportées par Pian *et al*., où un rôle significatif de la protéine Fhb dans la résistance à la phagocytose a été caractérisé. Dans cette dernière étude, les auteurs ont utilisé un modèle *in vitro* avec des leucocytes isolés de sang humain, alors que notre modèle utilise une lignée cellulaire de monocytes humains différenciés en macrophages. La différence de modèle pourrait en partie expliquer les différences rapportées. Néanmoins, aucun des mutants testés dans notre modèle ne démontre une plus grande susceptibilité à la phagocytose, suggérant un rôle minime des protéines Fhb et Fhbp dans la résistance à la phagocytose.

Finalement, nous avons testé la virulence du mutant simple de la protéine Fhbp dans un modèle *in vivo* murin puisque la virulence de ce mutant n'a jamais été évaluée. Nous n'avons observé aucune différence de virulence entre le mutant de la protéine Fhbp et la souche mère. Cependant, ces résultats sont à prendre avec précautions et conséquemment, n'ont pas été publié dans l'article principal sur le facteur H. Comme mentionné précédemment, au moment de la publication de notre article sur le facteur H, aucune donnée n'était disponible sur l'affinité des protéines Fhbp et Fhb pour le facteur H murin, ou de d'autres espèces. De plus, aucune source commerciale de facteur H murin complet (recombinant ou purifié) n'est disponible afin de tester ces hypothèses. À la suite de la publication de notre article sur le

recrutement du facteur H chez *S. suis* (**Article V**), une équipe a observé la présence de facteur H murin à la surface de *S. suis* incubé avec du sérum murin, sans toutefois confirmer quelle(s) protéine(s) est responsable de cette liaison[272]. Le rôle du facteur H murin dans la pathogenèse de *S. suis* reste ambigu.

Pour conclure cette section, nous avons démontré pour la première fois que le facteur H recruté par les protéines Fhbp et Fhb de *S. suis* représente un médiateur d'adhérence aux cellules épithéliales et endothéliales. Néanmoins, les rôles de ces protéines dans l'invasion, la résistance à la phagocytose et dans la virulence sont négligeables. Outre Fhb, Fhbp et la CPS, plusieurs autres facteurs semblent être capables de recruter le facteur H à la surface de *S. suis*[272]. Finalement, d'autres études seront nécessaires afin de déterminer quelles régions du facteur H interagissent avec les différents composants exprimés à la surface de *S. suis*.

V. CONCLUSIONS ET PERSPECTIVES

Dans le cadre de ce projet de thèse, nous avons établi les observations et perspectives suivantes:

Axe I. Rôle de la structure et de la composition de la CPS de *S. suis* sur ces propriétés fonctionnelles

- Nous avons tout d'abord caractérisé de nouveaux loci capsulaires (NCLs) chez *S. suis*. Ces nouveaux loci codent pour des gènes de synthèse de CPSs appartenant à de nouveaux types capsulaires. Ces loci codent potentiellement pour des CPSs antigéniquement (structures et compositions) différentes des autres sérotypes déjà caractérisés.

- Nous avons démontré que la majorité des souches non-sérotypables isolées au Québec (72%) possèdent un nouveau locus capsulaire (NCL1-20, Chz), dont certains que nous avons caractérisés pour la première fois (NCL17-20). Les NCLs caractérisés (NCLs 17-20) sont uniques aux souches isolées du Québec. Une petite proportion des souches non-sérotypables isolées du Québec correspondent à des sérotypes connus, mais l'absence, ou la faible production de la CPS, rend impossible le sérotypage.

- Nous avons démontré que, malgré les différences de composition et de structure, la CPS de *S. suis* sérotype 14 est un facteur antiphagocytaire et un facteur de virulence critique, tout comme chez le sérotype 2.

- Nous avons obtenu pour la première fois des mutants isogéniques de *S. suis* capables d'exprimer une CPS d'un sérotype différent. Ces mutants nous ont permis de démontrer que le changement de sérotype capsulaire n'influence pas la virulence d'une souche, et que le bagage génétique est plus important pour la virulence d'une souche.

Perspectives;

- La découverte de nouveaux NCLs ouvre la porte à plusieurs perspectives intéressantes;

  (i) D'un point de vue épidémiologique, il serait intéressant de déterminer si ces nouveaux NCLs retrouvés en Chine et au Québec sont retrouvés ailleurs dans le monde. Si nous considérons ces nouveaux NCLs (21) comme des sérotypes, *S. suis* comprendrait 50 sérotypes différents. La caractérisation de d'autres souches non-sérotypables provenant de d'autres pays (ou régions pour le Canada) pourrait sensiblement augmenter la diversité capsulaire de *S. suis* et se rapprocher de celle de *S. pneumoniae* (90 sérotypes).

  (ii) Pour le diagnostic, le développement des sérums contre la CPS de ces nouveaux sérotypes permettrait d'inclure ces sérotypes dans les tests diagnostiques.

  (iii) Pour la recherche fondamentale, la caractérisation de la composition et de la structure de ces CPSs permettrait de caractériser complètement ces nouvelles structures capsulaires.

- Finalement, les mutants isogéniques obtenus exprimant une CPS d'un sérotype différent représentent des outils très intéressants afin d'étudier l'effet de la composition de la CPS. Il pourrait s'avérer intéressant de comparer d'autres propriétés fonctionnelles de la CPS à l'aide de ces mutants, notamment le recrutement du facteur H et l'induction de cytokines par les cellules immunitaires.

Axe II. Rôle de l'acide sialique sur la structure et la synthèse de la CPS chez *S. suis,* en comparaison avec GBS

- En comparant avec GBS, nous avons démontré que la synthèse de la CPS de *S. suis* est très stricte et s'apparente davantage à la synthèse de la CPS de *S. pneumoniae*. Nous avons confirmé, grâce à plusieurs mutants, que l'acide sialique (sucre terminal d'une chaîne latérale) est crucial pour la synthèse de la CPS chez *S. suis* sérotypes 2 et 14. Il semble donc impossible d'obtenir des mutants encapsulés, mais sans acide sialique chez *S. suis* sérotypes 2 et 14.

- Chez GBS types III et V, nous avons démontré que la synthèse de la CPS en absence d'acide sialique est possible et semble donc plus permissive. En effet, nous avons démontré que le type V, comme le type III, est capable d'exprimer une CPS asialylée, mais également sialylée via un lien α2,6. La sialyltranférase de *S. suis* est donc capable de sialyler les chaînes polysaccharidiques de GBS types III et V.

- Ces observations contribuent à mieux comprendre l'influence de l'acide sialique sur la synthèse et la structure de la CPS polysaccharidique de *S. suis* et GBS.

Perspectives;

- Les mutants isogéniques obtenus représentent des outils très intéressants afin d'étudier l'effet du lien de l'acide sialique dans les propriétés immunogéniques de la CPS. Par exemple, il pourrait être intéressant de déterminer l'influence du lien de l'acide sialique (α2,6 vs α2,3) dans l'interaction avec les Siglecs présents à la surface de certaines cellules immunitaires. Les CPSs purifiées pourraient également être utilisées afin d'approfondir l'étude du rôle du lien de l'acide sialique dans le potentiel immunogénique de la CPS de GBS type III, ainsi que celle du type V.

Axe III. Rôle des protéines de surface ancrées à la paroi, Sao, Fhbp et Fhb, dans la pathogenèse de l'infection causée par *S. suis.*

- La synthèse de la protéine Sao n'est pas requise pour l'adhésion ni pour l'invasion des cellules de l'hôte et n'influence pas les propriétés antiphagocytaires et la virulence de *S. suis*. Par conséquent, une protéine de surface immunogénique n'est pas forcément un facteur de virulence critique. Néanmoins, on ne peut pas exclure que cette protéine exerce un rôle mineur dans la virulence.

- Nous avons démontré que le facteur H agit comme un médiateur d'adhérence, augmentant l'adhésion de *S. suis* aux cellules endothéliales porcines et aux cellules épithéliales humaines.

- Des mutants simples et doubles des protéines liant le facteur H, Fhb et Fhbp, ont permis de démontrer un rôle partiel de ces protéines dans l'adhésion en présence de facteur H. Il a été démontré que d'autres facteurs, dont la CPS, sont responsables du recrutement du facteur H à la surface de *S. suis*.

- Finalement, les protéines Fhb et Fhbp ne semblent pas être impliquées dans la résistance à la phagocytose.

- Ces observations contribue à mieux comprendre les mécanismes de colonisation de *S. suis* ainsi que la pathogenèse de l'infection causée par *S. suis*.

Perspectives;

- Chez *S. suis*, il existe plusieurs autres protéines de surface qui ont des rôles encore inconnus. Il est fort probable que plusieurs de ces protéines soient impliquées dans la colonisation et/ou dans la résistance au système immunitaire de l'hôte. Dans le **Tableau I** sur les facteurs de virulence, plusieurs protéines sont identifiées comme

ayant des mutants 'non disponibles'. Ces protéines représentent des cibles intéressantes pour l'obtention et la caractérisation de futurs mutants de *S. suis*.

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VII. ANNEXES

ANNEXE; ARTICLE VII

Characterization of *Streptococcus suis* isolates recovered between 2008 and 2011 from diseased pigs in Québec, Canada

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Publié dans: Vet. Microbiol. **2013**. 162: 819–825

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

Je suis coauteur de l'article. J'ai participé activement aux expériences et à l'analyse des résultats.

Abstract

In the present study we report the distribution of different serotypes of *Streptococcus suis* among strains isolated from diseased pigs in Québec, Canada, recovered between 2008 and 2011. Serotype 2 strains were further studied for the presence of the following virulence markers: suilysin (*sly*), muramidase-released protein (MRP), extracellular protein factor (*epf*) and the pilus encoded by the *srtF* cluster. Of 1004 field strains collected, 986 were confirmed to be *S. suis* by either the species-specific PCR targeting the gdh gene or by 16S rRNA gene sequencing analysis. Results showed that, although widely used, the species specific PCR test can sometimes be misleading and fail to correctly identify some *S. suis* isolates. Serotypes 2, 3, 1/2, 4, 8 and 22 together represented 51% of *S. suis* strains (64.5% of typable strains). Results confirmed the relatively low prevalence of serotype 2 in North America, when compared to European and Asian countries. The vast majority of serotype 2 field strains (96%) belong to either the MRP$^+$, *srtF* pilus$^+$, *epf*, *sly*$^-$ (52%) or the MRP$^-$, *srtF* pilus$^-$, *epf*, *sly*$^-$ phenotypes (44%). Most non-typable strains (89%) presented high surface hydrophobicity, suggesting that these are poorly or non-encapsulated. Electron microscopy studies confirmed the lack of capsular polysaccharide in selected non-typable high hydrophobic strains. The role and pathogenesis of the infection caused by these strains remain to be elucidated.

Introduction

Streptococcus suis is a major agent of meningitis, septicemia, and other diseases in pigs and is also a zoonotic agent [1]. A total of 35 serotypes have been described, based on a serological reaction against the capsular polysaccharide (CPS) [1]. Serotype 2 is considered the most virulent and the most frequently type associated with disease, mainly in Europe and Asia [1]. Interestingly, serotype 2 cases have been suggested in the past to be less prevalent in North America [2 3 4 5]. Other serotypes, such as serotypes 1/2, 5, 9 and 14, have also been associated with outbreaks in pigs in North America and Europe [1, 6]. Some *S. suis* isolates do not agglutinate with any of the typing antisera directed against the 35 serotypes (non-typable isolates) [3]. These strains may represent novel, yet undescribed, encapsulated serotypes or they may simply be non-encapsulated, naturally occurring mutant derivatives of known serotypes

and therefore impossible to serotype using the current typing scheme based on CPS antigens.

Different virulence factors of *S. suis* have been proposed [7], although many of them are present in both virulent and non-virulent strains [8]. Three virulence markers associated to *S. suis* serotype 2 have been historically used to differentiate virulent from non-virulent strains [9]. The suilysin (SLY, encoded by gene *sly*), a hemolysin which has a cytotoxic effect and two proteins of unknown function: the muramidase-released protein (MRP, encoded by gene *mrp*) and the secreted extracellular factor (EF, encoded by gene *epf*) [9, 10]. There is a positive association between the presence of MRP/EF/SLY and the virulence in Eurasian strains of *S. suis* [8]. However it has been suggested that many serotype 2 isolates recovered from diseased animals presented a $EF^-/SLY^-/MRP^{-/+}$ phenotype [4, 11]. Strains of serotype 2 may also express on their surface at least two different pili (encoded by the *srtF* and *srtG* pilus gene clusters) [12] [13]. A recent study from our laboratory with a limited number of strains from Canada and USA identified two prevalent lineages of *S. suis* serotype 2 strains, as characterized by multilocus sequence typing: sequence type (ST)25 and ST28, with a MRP^-/*srtF* $pilus^-$ and MRP+/*srtF* $pilus^-$ phenotypes, respectively [14]. In the present study we report the distribution of different serotypes among all strains isolated in Quebec, Canada, during four complete years, from 2008 to 2011. Serotype 2 strains were further studied for the presence of virulence markers *sly*, *efp* as well as the expression of MRP and the pilus encoded by *srtF* pilus cluster. Non-typable strains were also further characterized.

Material and methods

Isolation and identification of field strains of *S. suis* A total of 1004 field strains were collected from January 2008 to December 2011 from samples of diseased pigs that had been submitted to either the diagnostic service of the Faculty of Veterinary Medicine of the University of Montreal or the two official provincial laboratories. Tissue specimens were cultured on Tryptic Soy Agar (Difco Laboratories, Detroit, MI) with 5% bovine blood. Only streptococci isolated in pure culture or present in predominant number were submitted for further studies. Alpha-hemolytic colonies were subjected to a simplified biochemical

identification system where three tests were used to identify them as *S. suis*: no growth in 6.5% NaCl agar, a negative Voges-Proskauer test and positive amylase test [15] [16]. Serotyping was performed by the co-agglutination test as previously described [17].

Characterization of S. suis *serotype 2 strains*

Amplification of the *sly* and *epf* genes was performed by PCR as described previously [10]. Strains positive by PCR for sly and epf were further tested in a hemolysis assay and Western-blotting, respectively, as previously described [14]. Since correlation between PCR results for the gene mrp and the *srtF* gene cluster and actual expression of the proteins encoded by them has been reported to be sometimes inconsistent [4] [14], we only tested protein expression of the major pilin subunit Sfp1 and MRP by Western-blotting, as previously described [14] [18].

Characterization of non-typable strains

Each non-typable field strain was tested by a species specific PCR test targeting the *gdh* gene coding for the glutamate dehydrogensase [19]. PCR negative strains were sent for 16S rRNA gene sequencing analysis [20]. Hydrophobicity has previously been used as an indicator of the presence of capsule in *S. suis* strains [21] [22]. Non-typable PCR positive *S. suis* strains were further tested (triplicate independent assays) for cell surface hydrophobicity by measuring their absorption to nhexadecane according to the procedure previously described [21]. Two serotype 2 strains (reference strain S735 and strain AAH4) were used as positive controls. The absence of capsule was finally confirmed on selected strains by electron microscopy. Bacteria were grown overnight in THB medium, harvested by centrifugation, and washed once in PBS. The cells were fixed for 2 h at room temperature in 0.1 M cacodylate buffer (pH 7) containing 5% glutaraldehyde and 0.15% ruthenium red. They were then reacted with polycationic ferritin (1 mg/ml) and processed as previously described [21]. Thin sections were examined using a JEOL 1230 transmission electron microscope at an accelerating voltage of 60 kV.

Results and discussion

Confirmation of non-typable field strains as being S. suis

Results obtained in this study clearly demonstrate the convenience of a simplified biochemical identification system applied to strains recovered from diseased animals [15] [16]. Indeed, of a total of 1004 strains presumptively identified as *S. suis* by the diagnostic laboratory, 986 strains (98%) were clearly typable or, if non-typable, confirmed to belong to the *S. suis* species by either PCR or 16S rRNA sequencing (see below). All typable strains presented also a positive reaction with the *gdh* gene PCR. Of the 31 nontypable strains that tested negative for the species-specific *S. suis* PCR (in at least three independent assays), 12 (39%) were later confirmed to be *S. suis* by 16S rRNA gene sequencing analysis. These results indicate that, although widely used, the PCR test targeting the *gdh* gene [19] can sometimes fail to correctly identify certain *S. suis* isolates. Strains misidentified by the diagnostic laboratory as *S. suis* species were confirmed to be *Streptococcus orisratti*, *Streptococcus porcorum, Streptococcus porci, Streptococcus parauberis, Globicatella suldifac, Streptococcus spp.* or *Enterococcus spp*. Once these strains were eliminated, the actual *S. suis* sample was composed by the following number of strains per year 296 (2008), 268 (2009), 219 (2010) and 203 (2011).

Distribution of different serotypes among field strains of S. suis

Table I shows the distribution of different serotypes during the 4-year study. Although data are somehow comparable those of 2007 [3], some changes have been observed. In both 2008 and 2009, serotype 3 was, for the first time, the most prevalent serotype among *S. suis* isolated from diseased pigs, followed by serotype 2. Although serotype 2 became the serotype most frequently identified in the last two years, its prevalence remained relatively similar to that of serotype 3 (Table I). These data confirm the relatively low prevalence of serotype 2 in North America, when compared to European and Asian countries. The use of commercial bacterins (with serotype-specific protection and produced with serotype 2 isolates only) should be carefully evaluated after serotyping of strains affecting a given herd. Serotypes 1/2, 4, 8 and 22 have also been frequently identified during the four-year study.

Table I. Distribution of serotypes (number of isolates and percentage) of *Streptococcus suis* isolated from diseased pigs in Quebec, Canada, between 2008 and 2011.

Serotype	Year of isolation			
	2008	2009	2010	2011
1	4 (1.4%)	4 (1.5%)	3 (1.4%)	3 (1.5%)
2	38 (12.8%)	29 (11.0%)	39 (17.8%)	27 (13.4%)
1/2	32 (10.8%)	17 (6.3%)	19 (8.7%)	11 (5.4%)
3	43 (14.5%)	33 (12.3%)	23 (10.5%)	19 (9.4%)
4	15 (5.1%)	10 (3.7%)	11 (5.0%)	12 (5.9%)
5	10 (3.4%)	10 (3.7%)	7 (3.2%)	3 (1.5%)
6	2 (0.7%)	0 (0.0%)	0 (0.0%)	2 (1.0%)
7	14 (4.7%)	9 (3.4%)	8 (3.7%)	4 (2.0%)
8	27 (9.1%)	20 (7.5%)	17 (7.8%)	10 (5.0%)
9	6 (2.0%)	4 (1.5%)	5 (2.3%)	7 (3.5%)
10	2 (0.7%)	2 (0.7%)	0 (0.0%)	4 (2.0%)
11	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
12	1 (0.3%)	2 (0.7%)	2 (0.9%)	1 (0.5%)
13	2 (0.7%)	3 (1.1%)	2 (0.9%)	1 (0.5%)
14	2 (0.7%)	2 (0.7%)	5 (2.3%)	4 (2.0%)
15	0 (0.0%)	2 (0.7%)	0 (0.0%)	0 (0.0%)
16	4 (1.4%)	6 (2.2%)	4 (1.8%)	8 (4.0%)
17	2 (0.7%)	2 (0.7%)	1 (0.5%)	0 (0.0%)
18	3 (1.0%)	4 (1.5%)	0 (0.0%)	1 (0.5%)
19	4 (1.4%)	0 (0.0%)	5 (2.3%)	1 (0.5%)
20	1 (0.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
21	6 (2.0%)	4 (1.5%)	4 (1.8%)	2 (1.0%)
22	13 (4.4%)	18 (6.7%)	16 (7.3%)	6 (3.0%)
23	3 (1.0%)	3 (1.1%)	1 (.5%)	3 (1.5%)
24	1 (0.3%)	1 (0.4%)	1 (0.5%)	0 (0.0%)
25	1 (0.3%)	1 (0.4%)	0 (0.0%)	0 (0.0%)
26	1 (0.3%)	1 (0.4%)	0 (0.0%)	1 (0.5%)
27	5 (1.7%)	0 (0.0%)	1 (0.5%)	0 (0.0%)
28	1 (0.3%)	3 (1.1%)	2 (0.9%)	3 (1.5%)
29	1 (0.3%)	4 (1.5%)	3 (1.4%)	1 (0.5%)
30	2 (0.7%)	3 (1.1%)	1 (0.5%)	0 (0.0%)
31	0 (0.0%)	3 (1.1%)	1 (0.5%)	0 (0.0%)
32	0 (0.0%)	1 (0.4%)	0 (0.0%)	0 (0.0%)
33	5 (1.7%)	11 (4.1%)	1 (0.5%)	0 (0.0%)
34	2 (0.7%)	1 (0.4%)	0 (0.0%)	1 (0.5%)
NT	43 (14.5%)	55 (20.5%)	37 (16.9%)	67 (33.2%)

In fact, these four serotypes, plus serotype 2 and 3, represent 505 strains or 51% of *S. suis* strains (64.5% of typable strains). There is no clear association of a specific serotype and site of isolation from diseased animals (see Table II for the most important serotypes). In the past, serotype 1 strains were regularly recovered in Canada [23], but no serotype 1 strains were recovered in 2007 [3]. We report here that 14 strains (1.4%) belonging to this serotype were isolated between 2008 and 2011 and more than five additional strains have been recovered in 2012 (unpublished data).

Table II. Distribution of the most frequently *Streptococcus suis* serotypes and untypable strains recovered in Quebec, Canada, from 2008 to 2011 according to the tissues or organs from which they originated.

	No of isolates (percentage)					
Serotype	Multiple tissues[a]	Meninges/brain	Joints	Heart	Lungs	Others[b]
2	20 (15)	22 (16.5)	30 (22.5)	15 (11)	33 (25)	13 (10)
3	20 (17)	20 (17)	24 (20)	11 (9)	30 (26)	13 (11)
1/2	14 (18)	12 (15)	14 (18)	8 (10)	17 (21)	14 (18)
8	12 (16)	14 (19)	11 (15)	11 (15)	20 (27)	6 (8)
4	7 (15)	9 (19)	7 (15)	5 (10)	15 (31)	5 (10)
NT	30 (15)	36 (18)	28 (14)	26 (13)	55 (27)	27 (13)

These data may indicate the reemergence of this serotype, which has been described in previous studies as being highly virulent [24]. Changing of serotype prevalence with time in a given country has already been described [25, 26]. Other serotypes with a relatively high rate of isolation through the years are serotype 7 and 5 with 35 (3.5%) and 30 (3.0%) strains, respectively. Differences with Europe and Asia are also seen for serotypes other than serotype 2. In fact, we only identified a small percentage (1.3%) of serotype 14 strains. This latter is considered an important serotype in United Kingdom and an important zoonotic agent in Thailand [27 28]. Only 2.2% of isolates were serotype 9, which is one of the most prevalent serotypes in some European countries [6, 26]. Serotype 11 was the only serotype which has not been isolated in this study, although it is present in Canada, with 3 previous isolates recovered in 2007 [3]. While isolates belonging to serotypes 20, 26 and 32 had not been isolated between 2001 and 2007 [3], we report here their isolation in Canada between 2008 and 2011. Serotyping remains a valuable tool used by veterinary practitioners and diagnosticians to understand the epidemiology of a particular outbreak and/or to increase the possibility of success of a vaccination program within a herd [1 15].

Characterization of* S. suis *serotype 2 field isolates

The presence of genes coding for the suilysin (*sly*) and the EF (*epf*) were tested by PCR in all serotype 2 strains and positive strains were confirmed to express the proteins by the hemolysis or western-blotting assays, respectively. Tests were carried out on 127 serotype 2 strains that were still viable. A vast majority of serotype 2 strains studied here (96%) belonged to one of the two following phenotype groups: 52% presented a profile MRP$^+$, expression of *srtF* pilus$^+$, *epf*$^+$, *sly*$^-$ and 44% presented a profile MRP$^-$, expression of *srtF* pilus$^-$, *epf*$^+$, *sly*$^-$ (Table III). Special antigenic and/or virulence factor characteristics of these two groups of strains are still unknown, although it has been reported a higher virulence potential for strains of the MRP$^-$, expression of *srtF* pilus$^-$, *epf*$^+$, *sly*$^-$ phenotype in comparison to those with MRP$^+$, expression of *srtF* pilus$^+$, *epf*$^+$, *sly*$^-$ profile [14].

Table III. Relationship between *Streptococcus suis* serotype 2 strains recovered in Quebec, Canada, from 2008 to 2011 and the presence of muramidase-released protein (MRP), extracellular factor (*epf*), suilysin (*sly*) and *srtF* pilus cluster.

Year	Number of strains	Presence of markers			
		MRP$^+$/*srtF* pilus$^+$/*epf*$^-$/*sly*$^-$	MRP$^-$/*srtF* pilus$^-$/*epf*$^-$/*sly*$^-$	MRP$^-$/*srtF* pilus$^+$/*epf*/*sly*$^-$	MRP$^+$/*srtF* pilus$^+$/*epf*$^+$/*sly*$^+$
2008	34	17	17	0	0
2009	27	16	11	0	0
2010	39	21	16	1	1
2011	27	12	12	2	1
Total	127	66	56	3	2

Two strains presented the typical Eurasian profile of MRP$^+$/expression of *srtF* pilus$^+$/*epf*$^+$/*sly*$^+$ (Table III) [14]. Both strains presenting the *sly*$^+$ and *epf*$^+$ genotypes were confirmed to induce hemolysis using horse red blood cells and protein expression by Western-blotting, respectively. On the basis of its low frequency of isolation, it can be speculated that these ‘‘Eurasian-type strains’’ were introduced in North America by importation of animals. Human travel might also contribute to dissemination of these potentially highly virulent strains, as exemplified by a reported case of human *S. suis* meningitis caused by this type of strain (unpublished data) involving a patient who contracted the infection in the Philippines but in whom clinical signs appeared only after he returned to the USA [29]; however,

transmission of the infection from humans to pigs have never been described. Maintaining a low prevalence of these strains among the swine population in North America is crucial for animal and human health. Of note, the only locally acquired human infection in the USA described so far [29, 30] was caused by a ST1 strain with a typical MRP$^+$/expression of *srtF* pili$^+$/*epf*$^+$/*sly*$^+$ (M. Gottschalk, unpublished data). Finally, three strains presented the atypical profile MRP$^-$/expression of *srtF* pilus$^+$/epf$^-$/sly$^-$ (Table III). It has been reported that certain strains had truncations or point mutations in the mrp gene that prevented expression of MRP protein, which may explain results obtained with these strains.

Characterization of non-typable strains

Non-typable *S. suis* strains may correspond to either true encapsulated strains that belong to novel, not yet described serotypes or to non-encapsulated strains, which are impossible to serotype using the current typing methods. A total of 174 non-typable strains were viable and available for further characterization (Table IV).

Table IV. Hydrophobicity of non-typable *Streptococcus suis* strains recovered from diseased pigs in Quebec, Canada, from 2008 to 2011.[a]

Year of isolation	Number of strains	Low hydrophobicity[b]	High hydrophobicity[c]
2008	30	4	26
2009	47	5	42
2010	37	3	34
2011	60	7	53
Total	174	19	155

[a] Cell surface hydrophobicity test was performed by measuring absorption to n-hexadecane (Bonifait et al., 2010).
[b] ≤30% of hydrophobicity.
[c] ≥70% of hydrophobicity

Strains were divided in those with high hydrophobicity (≥70%) and those with low hydrophobicity (≤30%). A total of 155 strains (89% of non-typable strains tested) presented high hydrophobicity. It has been shown that *S. suis* strains with high hydrophobicity are most

probably poorly or nonencapsulated [21]. Six representative strains with high hydrophobicity were observed by electron microscopy using the ferritin staining method to confirm whether they indeed did not possess capsule.

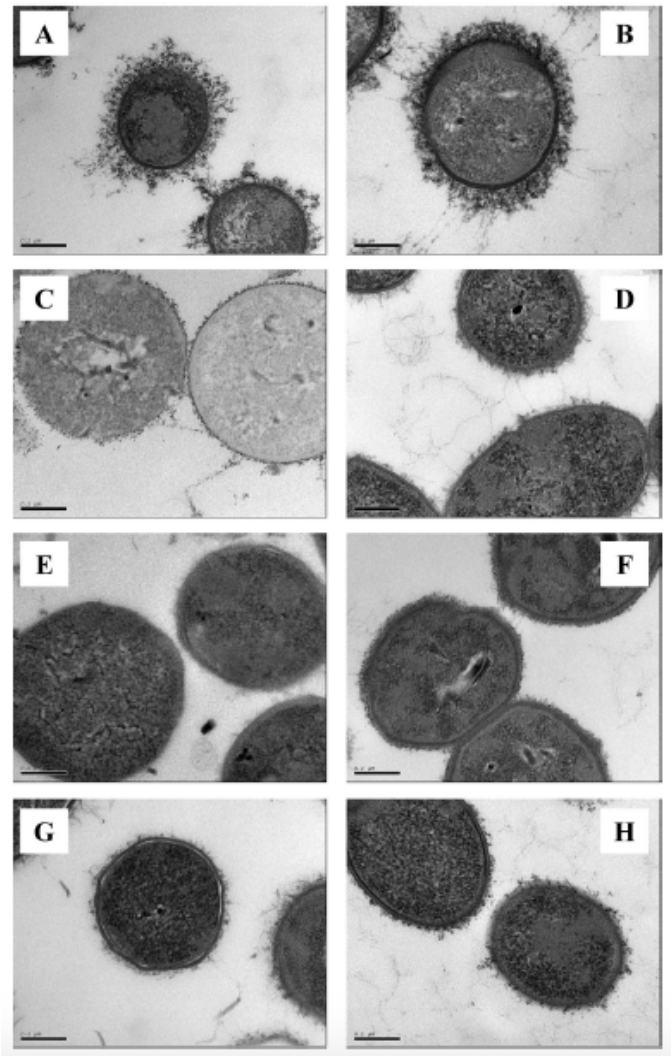

Figure 1. Visualization of *Streptococcus suis* capsule by transmission electron microscopy following ferritin stabilization. (A) *S. suis* S735 reference strain, serotype 2 (4% of hydrophobicity); (B) *S. suis* strain AAH4, serotype 2 (3% of hydrophobicity); (C) *S. suis* 1151109 (non-typable strain, 74% of hydrophobicity); (D) *S. suis* 1148795 (non-typable strain, 93% of hydrophobicity); (E) *S. suis* 1145879 (non-typable strain, 81% of hydrophobicity); (F) *S. suis* 1079506 (nontypable strain, 92% of hydrophobicity); (G) *S. suis* 1140322 (non-typable strain, 89% of hydrophobicity); (H) *S. suis* 1077009 (non-typable strain, 86% of hydrophobicity). Bar: 0.2 μm.

TEM showed that all six strains were non-encapsulated (Fig. 1), whereas the reference strain S735 and strain AAH4 of serotype 2 clearly showed the capsular polysaccharide on the cell surface (Fig. 1) and presented very low hydrophobicity (4%). Further studies are needed to confirm whether or not some of these non-encapsulated strains belong to well know serotypes. It is also difficult to ascertain if these strains were already non-encapsulated when causing disease or if they lost the capsular polysaccharide during isolation and culture. It has been previously reported that 34% of isolates belonging to serotype 2 or 1/2 and recovered from cases of endocarditis in Japan were non-encapsulated due to deletions and insertions in genes of the capsular polysaccharide locus [31]. It was concluded that although the capsule is considered to be an important virulence factor in *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 higher adherence to mammalian cells but also a capacity to form biofilms [21]. Since the site of isolation of these non-typable strains was similar to those of most important serotypes found in this study (Table II), their potential virulence capacity should not be ruled out.

Conclusion

As a conclusion, the distribution of different serotypes recovered from diseased pigs in Canada shows a proportionally low prevalence of serotype 2 and an increased importance of serotype 3. Serotype 2 strains are mostly divided in two phenotypic groups. Finally, most nontypable strains are non-encapsulated. Studies on the virulence and pathogenesis of the infection caused by these non-encapsulated strains should be carried out.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant #154280 as well as NSERC Discovery Accelerator Supplement #380299 to MG as well as internal funds from the diagnostic service of the Faculty of Veterinary Medicine of the University of Montreal.

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ANNEXE; ARTICLE VIII

Implication of TLR- but not of NOD2-signaling pathways in dendritic cell activation by Group B *Streptococcus* serotypes III and V

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Publié dans: PLoS One. **2014**. 9(12): e113940. doi: 10.1371/journal.pone.0113940

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

Je suis coauteur de l'article. J'ai construit la souche mutante non encapsulée de GBS type V utilisée dans les expériences de cette articles.

Abstract

Group B *Streptococcus* (GBS) is an important agent of life-threatening invasive infection. It has been previously shown that encapsulated type III GBS is easily internalized by dendritic cells (DCs), and that this internalization had an impact on cytokine production. The receptors underlying these processes are poorly characterized. Knowledge on the mechanisms used by type V GBS to activate DCs is minimal. In this work, we investigated the role of Toll-like receptor (TLR)/MyD88 signaling pathway, the particular involvement of TLR2, and that of the intracellular sensing receptor NOD2 in the activation of DCs by types III and V GBS. The role of capsular polysaccharide (CPS, one of the most important GBS virulence factors) in bacterial-DC interactions was evaluated using non-encapsulated mutants. Despite differences in the role of CPS between types III and V GBS in bacterial internalization and intracellular survival, no major differences were observed in their capacity to modulate release of cytokines by DC. For both serotypes, CPS had a minor role in this response. Production of cytokines by DCs was shown to strongly rely on MyD88-dependent signaling pathways, suggesting that DCs recognize GBS and become activated mostly through TLR signaling. Yet, GBS-infected $TLR2^{-/-}$ DCs only showed a partial reduction in the production of IL-6 and CXCL1 compared to control DCs. Surprisingly, CXCL10 release by type III or type V GBS-infected DCs was MyD88-independent. No differences in DC activation were observed between $NOD2^{-/-}$ and control DCs. These results demonstrate the involvement of various receptors and the complexity of the cytokine production pathways activated by GBS upon DC infection.

Group B *Streptococcus* (GBS) or *Streptococcus agalactiae* is an important cause of severe invasive bacterial infections worldwide (1). 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 (2). Clinical isolates of GBS are covered by a capsular polysaccharide (CPS) recognized as the most important factor for bacterial survival within the host (3). Among ten GBS CPS types

that have been characterized (1, 2, 4), type III GBS is the most common type in GBS meningitis (1). A cohort study suggested that the high invasiveness of this serotype may be related, at least in part, to inadequate maternal and infant levels of type III CPS-specific antibodies (5). Type V GBS has long been recognized as a leading cause of invasive disease in adults (2, 6). To date, there are no guidelines for the prevention of adult GBS disease; vaccines in development may hold promise (6). In a cross-sectional study analyzing older adult subjects (7), impaired type V GBS killing was associated with a low concentration of CPS-specific antibodies as well (7, 8).

Different types of leukocytes accomplish dedicated tasks in antigen presentation and killing of pathogens (9). Dendritic cells (DCs) are recognized as the most powerful antigen-presenting cells (APCs) that initiate immune responses against pathogens and are considered an essential link between innate and adaptive immunity. In fact, DCs principal function is to alert the immune system, not to clear invading microorganisms. DCs capture and process antigens, and then undergo a maturation process characterized by the production of cytokines and up-regulation of co-stimulatory molecules. Afterwards, DCs migrate to adjacent lymphoid organs where they activate T cells (10). The interactions between DCs and pathogens can, not only influence the pathogenesis of a disease, but also the magnitude and phenotype of the ensuing adaptive immune response. Recognition of pathogen-associated molecular patterns (PAMPs) by DCs is mediated by pattern-recognition receptors (PRRs), including the Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) families (11). Most TLRs are transmembrane proteins for sensing extracellular pathogens whereas NLRs sense PAMPs in the cytosolic compartment. Specially, TLR2 is reported to be specialized for the recognition of lipoproteins by generally forming a heterodimer with TLR1 or TLR6 (12, 13). NLRs include the two-well characterized NOD1 and NOD2 (14). NOD2 is known to sense molecules produced during the synthesis and/or degradation of bacterial peptidoglycan (PGN) and recognize muramyl dipeptide (15), a PGN constituent of both Gram-positive and Gram-negative bacteria. Interactions between TLRs and NODs with their ligands initiate an intracellular signaling cascade that induces the secretion of several pro-inflammatory cytokines and the expression of co-stimulatory cell surface molecules through the activation of transcription factors, including NF-κB (13). Signaling occurs through

association of TLRs with several adaptor molecules, such as the myeloid differentiation factor 88 (MyD88) (13). MyD88 is utilized by all TLRs with the exception of TLR3 and drives NF-κB and mitogen-activated protein kinase (MAPK) activation to control inflammatory responses.

In contrast to other Gram-positive bacteria, TLR2 seems to play a minor role in type III GBS interactions with the host (16, 23). Nevertheless, among immune cells, few studies explored the role of TLRs on GBS modulation of DC functions. It has been shown that despite the importance of MyD88, TLRs 2, 4 and 9 are not involved in the production of interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) by type III GBS-infected DCs (24). However, TLR7 and TLR9 do recognize type III GBS nucleic acids in DC phagolysosomes after partial bacterial degradation, leading to interferon-β secretion (IFN-β) (25). The few *in vitro* studies performed so far failed to demonstrate a clear role of NOD in types Ia and V GBS interactions with macrophages (23, 26). We previously reported that NOD2 is not a crucial receptor to fight type III GBS infection in adult mice but the release of inflammatory cytokines in sera and by total spleen cells from GBS-infected $NOD2^{-/-}$ mice is reduced (27). However, no data are available concerning the role of TLR2 in GBS induction of other cytokines and chemokines, or whether different GBS serotypes interact differently with DCs. Similarly, the role of NOD2 in GBS interactions with DCs is so far unknown. In this study, we used C57BL/6 mouse bone marrow-derived DCs (bmDCs) to investigate their interactions with types III and V GBS and evaluated the capacity of these different GBS serotypes to activate DC. The potential contribution of TLR2, MyD88 adaptor protein and NOD2 in this response was assessed using bmDC generated from knock-out (KO) mice. Finally, as CPS is the most external layer at the surface of the bacteria and thus a key candidate to be involved in bacterial internalization by and interaction with DCs, the role of bacterial CPS in these processes was studied using isogenic non-encapsulated mutants.

Materials and Methods

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 by the Animal Welfare Committee of the Université de Montréal. The protocols and procedures were approved by the Ethics Committee (CÉUA).

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in **Table I**. Encapsulated type III GBS strain COH-1 and its isogenic non-encapsulated mutant (Δ*cpsE*) were described in previous work (25-30). Strain CJB111 (ATCC BAA-23) is a highly encapsulated type V GBS isolate from a neonate with septicemia (31). An isogenic non-encapsulated type V (Δ*cpsE*) mutant was generated in this study (see below). GBS strains were grown in Todd-Hewitt Broth (THB) or agar (THA) (Becton Dickinson, Mississauga, ON, Canada) or on sheep blood agar plates at 37°C for 18 h. *Escherichia coli* strains were cultured in Luria-Bertani broth or agar (Becton Dickinson) at 37°C for 18 h. When necessary, antibiotics (Sigma-Aldrich, Oakville, ON, Canada) were added to culture media at the following concentrations: kanamycin and spectinomycin at 50 µg/ml for *E. coli*, and spectinomycin at 200 µg/ml for GBS. To perform GBS-bmDCs interaction studies, GBS strains were grown as previously described (28, 29) and diluted in complete cell culture medium prior to the experiments. 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, USA). Levels of CPS production by GBS type III and type V strains were compared by chromatographic purification methods and after purity confirmation by nuclear magnetic resonance as previously described (32). From 8 liters of GBS type III or type V culture (adjusted at O.D. values =0.8) the average yield of highly pure CPS was of: 59.25 mg ±17.5 (n=4) for type III GBS and of 50.5 mg ±9.1 (n=4) for type V GBS.

Table I. Bacterial strains, plasmids and oligonucleotide primers used in this study.

Strains/Plasmids/Primers	General characteristics	Source/Reference
Escherichia coli		
TOP 10	F-*mrcA Δ(mrr-hsd*RMS-*mcr*BC)*φ80 lacZΔM5 Δlac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Life Technologies Inc.
Group B *Streptococcus*		
COH-1	Wild-type, highly encapsulated strain isolated from an infant with bacteremia. Serotype III	[30]
Δ*cpsE*	Non-encapsulated strain derived from strain COH-1. Deletion of the *cpsE* gene	[28]
CJB111	Wild-type, highly encapsulated strain isolated from a neonate with septicemia. Serotype V	[31]
Δ*cpsE*	Non-encapsulated strain derived from strain CJB111. Deletion of the *cpsE* gene	In this work
Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Life Technologies Inc.
pSET4s	Thermosensitive vector for allelic replacement. Replication functions of pG+host3 and pUC19, MCS, *lacZ*, Sp^R	[33]
p4ΔcpsE	pSET4s carrying the construct for *cpsE* allelic replacement	In this work
Oligonucleotide primers, sequence (5′ – 3′)		
SAVE-ID1	*CGGGTTTATTGTTGGTGCAGG*	
SAVE-ID2	*TCTTCAAGATAGCCACGACTCC*	
SAVE-ID3	*GCGACGCCTTAGTTTTAAGCC*	
SAVE-ID4	*ACGGACGATTCATCATTCCCTC*	
SAVE-ID5	*TGGTCGTTCCTTCAGGGAAAG*	
SAVE-ID6	*GCTCCTGTCCCGAGTAAAACTCCACAACTGTTTGAATCATCGC*	
SAVE-ID7	*GCGATGATTCAAACAGTTGTGGAGTTTTACTCGGGACAGGAGC*	
SAVE-ID8	*AATGGTACTGCTACAGCGGC*	

Construction of type V GBS non-encapsulated mutant

GBS genomic DNA was extracted by InstaGene Matrix solution (BioRad Laboratories, Hercules, CA, USA). Minipreparations of recombinant plasmids and transformation of *E. coli* were performed by standard procedures. 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 iProof proofreading DNA polymerase (BioRad Laboratories, Mississauga, ON, Canada). Oligonucleotide primers were from IDT technology (Coralville, IA, USA). Amplification products were purified with QIAgen PCR purification kit (QIAGEN, Valencia, CA, USA) and sequenced with an ABI 310 automated DNA sequencer, using the ABI PRISM dye terminator cycle sequencing kit (Life Technologies Inc., Burlington, ON, Canada).

The DNA sequence of type V GBS strain CJB111 capsular (*cps*) locus was retrieved from GenBank (Accession numbers AAJQ01000091.1 and AAJQ01000044.1) and used as

sequence template for primers design. Precise deletion of the *cpsE* (1304 bp) gene was constructed by using splicing-by-overlap-extension PCR and the primers listed in **Table I**. The PCR-generated Δ*cpsE* deletion allele was cloned into plasmid pCR2.1 (Invitrogen), extracted with EcoRI and recloned into the thermosensitive shuttle plasmid pSET4s (33) digested with the same enzyme, giving rise to the knockout vector p4ΔcpsE. Electroporation of GBS with the recombinant plasmid and procedures for isolation of mutants were those described previously (28). Allelic replacement was confirmed by PCR and sequencing analysis. The non-encapsulated (Δ*cpsE*) phenotype of the mutant was confirmed by absence of reaction in the coagglutination test using rabbit antisera against type V GBS capsular material (Denka Seiken, Campbell, CA, USA), and by transmission electron microscopy using polycationic ferritin labeling as previously described (**Fig. 1A**) (28). Growth rates were not significantly affected in the mutant strain compared to wild-type (WT) GBS (**Fig. 1B**).

Generation of Bone Marrow-derived Dendritic Cells (bmDCs)

BmDCs were generated from six to eight week-old female mice originated from Jackson Laboratory (Bar Harbor, ME, USA), including control (CTRL) C57BL/6J, MyD88$^{-/-}$ (B6.129P2-*Myd88*^{tm1Defr}/J), TLR2$^{-/-}$ (B6.129-*Tlr2*^{tmlKir}/J) and NOD2$^{-/-}$(B6.129S1-*Nod2*^{tm1Flv}/J). BmDCs were produced according to a technique described elsewhere (28, 29). Briefly, after red blood cell lysis, total bone marrow cells (2.5×10^5 cells/ml) were cultured in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 20 µg/ml gentamicin, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine and 50 µM 2-mercaptoethanol. All reagents were from Gibco (Life Technologies Inc.). Complete medium was complemented with 20% GM-CSF from a mouse GM-CSF transfected cell line (Ag8653) as a source of GM-CSF (34). Cells were cultured for 7 days at 37°C with 5% CO_2. On day 7, clusters were harvested and subcultured overnight to remove adherent cells. Non-adherent cells were collected on day 8 and used as immature bmDCs for the studies. Cell purity was routinely ≧88% CD11c^{+high} cells as determined by FACS analysis and as previously reported (28)

Bacterial internalization assays

BmDCs at a concentration of 10^6 cells/ml were infected with 10^6 CFU/ml of WT type V GBS or its non-encapsulated mutant strain (initial MOI:1), and incubated for 0.5 to 3 h at 37°C with 5% CO_2 (without addition of exogenous complement). MOI and assay conditions were chosen based on previous studies on the kinetics of type III GBS phagocytosis by DCs (28). After incubation, 100 µg/ml of gentamicin and 5 µg/ml of penicillin G (Sigma-Aldrich) were added to kill extracellular bacteria. After 1 h-antibiotic treatment, cells were washed 3 times with PBS, lysed with sterile water and viable intracellular streptococci enumerated by quantitative plating of serial dilutions of the lysates on THA. For intracellular survival studies, internalization assays were performed as described above, except that after a 60 min initial bacterial-cell contact, gentamicin-penicillin were added and the treatment was lengthened for different times up to 5 h. The results were expressed as CFU/ml of recovered intracellular viable bacteria.

In vitro bmDC stimulation assay

BmDCs were resuspended at 10^6 cells/ml in complete medium and stimulated with different GBS strains (10^6 CFU/ml; initial MOI:1). Conditions used were based on those already published (28). After 2 h of bmDC-GBS infection, the bacteriostatic agent chloramphenicol (CM, 12 µg/ml, Sigma-Aldrich) was added to the culture to prevent cell toxicity as previously reported (28). After 16 h of incubation, supernatants were collected for cytokine quantification by ELISA. Non-stimulated cells served as negative control. The specific TLR2-ligand PAM(3)CSK(4) (at 0.5 µg/ml (Invivogen, San Diego, CA, USA)), the TLR4-ligand lipopolysaccharide (ultra-pure LPS, at 1 µg/ml (Apotech Corporation, Epalinges, Switzerland)), and the specific NOD2-ligand muramyl dipeptide (MDP, at 10 µg/ml (Invivogen)) were used as controls (**Fig. S1**). Lactate dehydrogenase (LDH) release measurement assay was used to confirm absence of cytotoxicity in bacterial-bmDC cultures (Promega CytoTox96, Promega Corporation, Madison, WI, USA) (28).

Cytokine quantification by ELISA

Levels of IL-6, IL-10, IL-12p70, IL-23p19, TNF-α, CCL2 (MCP-1), CCL3 (MIP-1α) CXCL1 (KC) and CXCL10 (IP-10) in cell culture supernatants were measured by sandwich ELISA using pair-matched antibodies from R&D Systems (Minneapolis, MN, USA) or eBioscience (San Diego, CA, USA), according to the manufacturer's recommendations. Twofold 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.

Statistical analysis

All data are expressed as mean ± SEM. Data were analyzed for significance using ANOVA combined with Bonferroni t-test for correction. A P value <0.05 was used as a threshold for significance. All experiments were repeated at least three times.

Results and discussion

DCs efficiently internalize both the encapsulated and the non-encapsulated type V GBS strains

We demonstrated previously that encapsulated type III GBS is easily internalized by DCs, but efficiently survives inside these cells for at least 6 h (28). The CPS only slightly and transitorily affects type III GBS internalization at short incubation times (1 h). On the other hand, the type III CPS plays a role in modulating bacterial survival within DCs (28). To date, despite interactions with human epithelial and endothelial cells (35-39), mouse macrophages or human neutrophils (23, 40-42), no data are available on the phagocytosis capacity of bmDCs face to type V GBS or on the importance of the type V CPS in modulating this activity. As shown in **Fig. 1C**, the encapsulated CJB111 strain was rapidly internalized by bmDCs. Phagocytosis levels increased over time reaching levels as high as 10^7 CFU of total recovered intracellular bacteria at 3 h. Longer incubation times could not be tested, as GBS

type V was toxic to bmDCs (by LDH assay, data not shown). After 1 h of incubation, the Δ*cpsE* mutant was significantly more internalized than the WT strain and this difference was observed at least until 3 h of incubation ($P<0.01$). Survival assay (**Fig. 1D**) showed a slow decrease in WT GBS intracellular survival, which was statistically significant after 1 h of GBS post-internalization ($P<0.01$), with ~2×10^3 CFU of remaining live intracellular bacteria at the end of the survival test. Although the non-encapsulated mutant strain was more internalized than the WT strain, the intracellular survival rates remained similar to those of the parental strain.

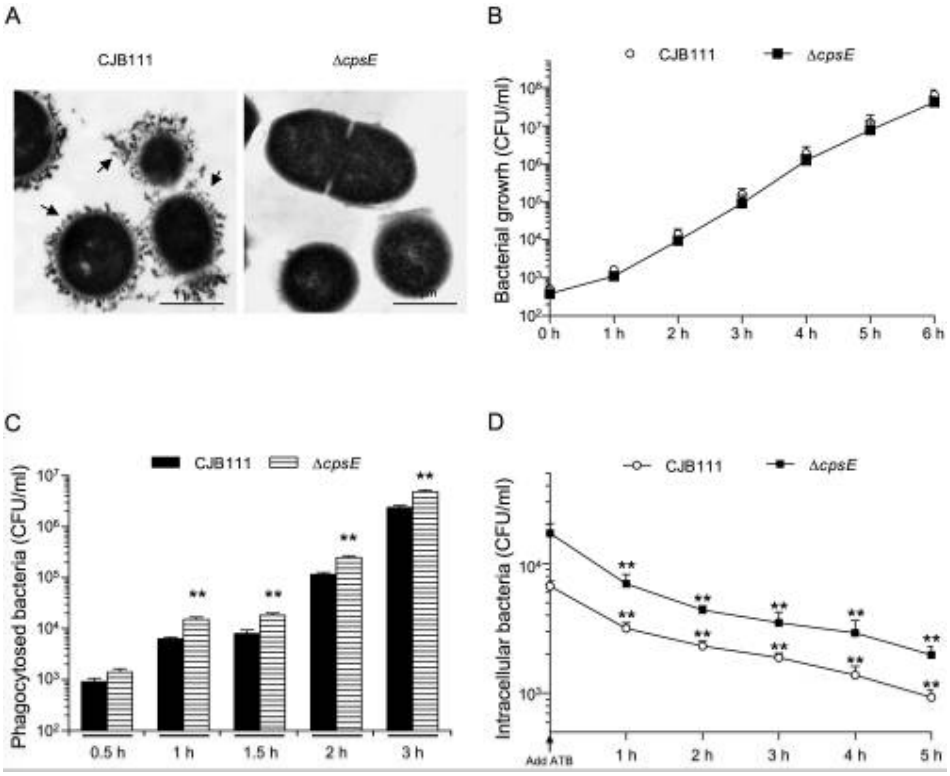

Figure 1. Phagocytosis by and intracellular survival within DCs of type V GBS: role of bacterial capsular polysaccharide. (A) Transmission electron micrographs of GBS strains labeled with polycationic ferritin show GBS wild-type strain CJB111 with a thick capsule (indicated by arrows) whereas no capsular material is observed in Δ*cpsE* mutant strain. (B) Growth curves of wild-type GBS strain CJB111 and Δ*cpsE* mutant strain. (C) Wild-type type V GBS strain CJB111 or the Δ*cpsE* non-encapsulated mutant (10^6 CFU/ml, initial MOI:1) were incubated with C57BL/6J-derived bmDCs for different time periods. Internalized bacteria were enumerated by quantitative plating after 1 h of antibiotic treatment to kill extracellular bacteria. ** $P<0.01$, indicates statistically significant differences between the wild-type strain CJB111 and the non-encapsulated mutant, n=5. (D) For intracellular survival assays, bmDCs were infected with GBS strains (MOI:1) and phagocytosis was left to proceed for 60 min. Antibiotics (ATB) were then added for 1 h (defined as time 0). This initial

antibiotic-treatment was extended up to 5 h and cells lysed to quantify intracellular bacteria by viable plate counting. ** $P<0.01$, indicates incubation times for which significantly differences in the numbers of recovered intracellular bacteria were observed compared to time 0, n=8. All results are expressed as CFU recovered bacteria per ml (means ± SEM). It should be noted that initial MOI was the same for all conditions and bacterial growth rate in the culture medium was identical for both strains.

Compared to type III GBS, type V CPS slightly prevented the internalization of GBS overtime, but does not seem to protect bacteria from intracellular killing. GBS is serologically classified into ten distinct serotypes (Ia, Ib, II-IX) based on antigenic differences in the CPS (3). The structures of the ten CPS types are similar in their constituent monosaccharide compositions and certain structural motifs, yet they differ sufficiently to be antigenically distinct. The polysaccharide repeat unit structures of types III and V are not closely related and may explain the observed differences (3). Variations in the thickness of CPS expressed by the strains used in this study cannot be completely ruled out, albeit CPS yields seem to be similar between the two strains (as aforementioned). Finally, intrinsic differences related to these two particular strains and independent of the serotype might also influence bacterial phagocytosis.

Internalization of GBS is independent of TLR and NOD2 receptor signaling

Albeit controversial, it has previously been reported that TLRs may be involved as receptors for bacterial phagocytosis (43, 44). The absence of TLR2 delayed *Streptococcus pneumoniae* phagocytosis and killing by neutrophils (45). In contrast, TLRs were shown not to play a significant role in phagocytosis of type III GBS by peritoneal macrophages (20) and *Streptococcus suis* by DCs (46). On the other hand, it has been observed that NOD1- or NOD2-deficient mice have decreased phagocytic abilities (47, 48), in particular in neutrophils. Yet, the influence of NOD receptors on phagocytosis does not seem to be universal (49, 50). So, in this study, GBS internalization by DCs was evaluated by the antibiotic-protection phagocytosis assay using bmDCs from MyD88$^{-/-}$, TLR2$^{-/-}$ and NOD2$^{-/-}$ mice. As displayed in **Fig. 2**, no significant differences were observed in bmDC capacity to internalize WT GBS strains in the absence of MyD88, TLR2 or NOD2. The difference in phagocytosis levels between WT strains and their respective non-encapsulated mutants remained the same in deficient-bmDCs compared to CTRL cells. Similarly, no differences

were observed in the intracellular fate of GBS strains between CTRL and KO cells (data not shown).

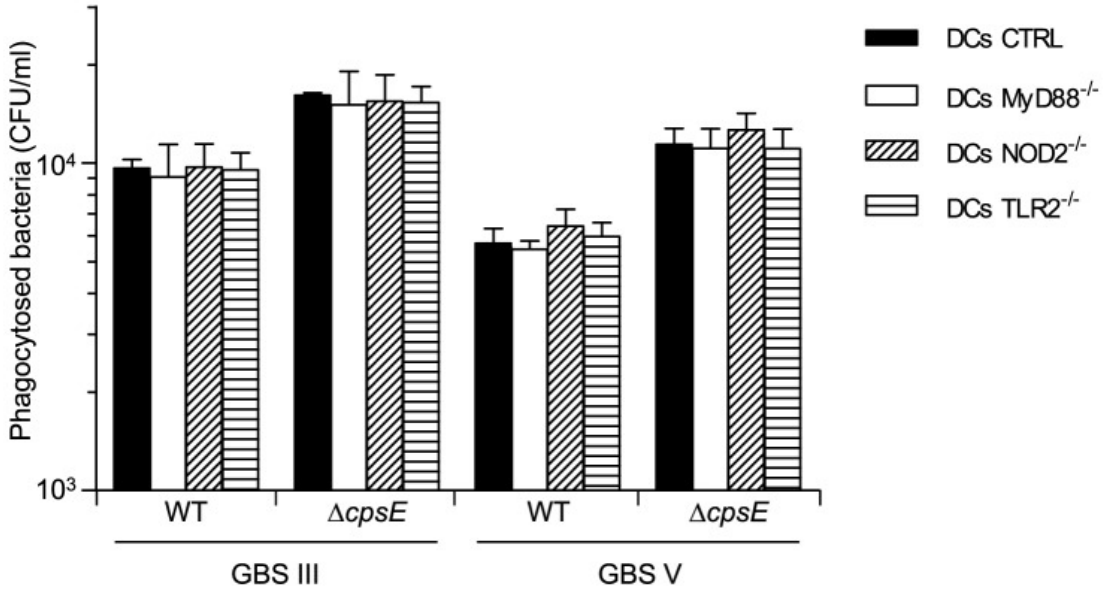

Figure 2. Effect of MyD88, TLR2 or NOD2 deficiency on the capacity of DCs to internalize GBS. Control (CTRL), MyD88$^{-/-}$, TLR2$^{-/-}$ or NOD2$^{-/-}$ bmDCs were incubated for 1 h with GBS wild-type (WT) strains or their respective non-encapsulated (Δ*cpsE*) mutants (10^6 CFU/ml, initial MOI:1). Internalized bacteria were enumerated by quantitative plating after 1 h of antibiotic treatment to kill extracellular bacteria (n=5).

DC Activation by GBS infection and the importance of MyD88 adaptor protein is not serotype restricted

As MyD88 adaptor protein mediates numerous biologically important signal transduction pathways in innate immunity (51), its contribution to DC cytokine production following stimulation with GBS was investigated. BmDCs from CTRL or MyD88$^{-/-}$ mice were incubated with different GBS strains for 16 h. Optimal assay conditions were chosen based on previous results (28) and preliminary studies on the kinetics of cytokine release by bmDCs in response to GBS (data not shown). As we previously reported (28, 29), type III GBS-activated DCs produced significant levels of the pro-inflammatory cytokines IL-6 and TNF-α, the Th1-driving cytokine IL-12p70, the regulatory cytokine IL-10, and the chemokines CCL2, CXCL1

and CXCL10 (**Fig. 3**). The encapsulated type V GBS strain released similar levels of these cytokines than the encapsulated type III GBS strain. BmDCs were also able to produce significant amounts of the Th17-driving cytokine IL-23 and high levels of another member of the CC chemokine family, CCL3, after exposure to either types III or V GBS. In general, the non-encapsulated mutants produced similar levels of cytokines and chemokines compared to respective WT strains (**Fig. 3**). Yet, the absence of CPS resulted in higher levels of IL-10 and IL-23 production by bmDCs stimulated with either serotype. We previously reported that the absence of CPS affects CCL2 production by type III GBS-infected DCs (29). The same phenotype was observed here for type V GBS. Furthermore, CCL3 production was also significantly attenuated in the non-encapsulated mutant strains for both GBS serotypes (**Fig. 3**). Thus, types III and V GBS induce similar patterns of cytokine production by DCs and this response is similarly affected by the presence of CPS in both serotypes.

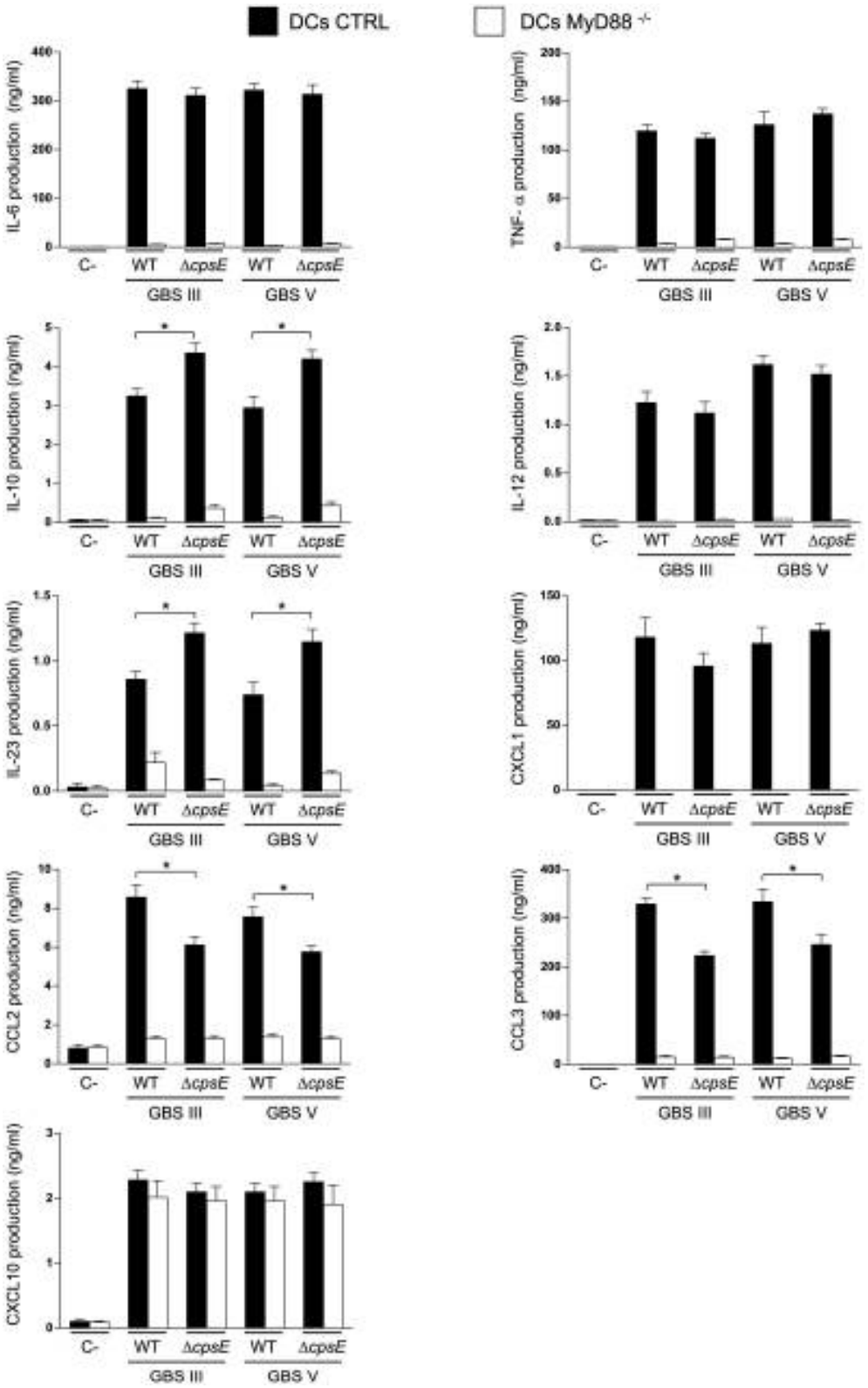
DCs CTRL
DCs MyD88 -/-
IL-6 production (ng/ml)
TNF- α production (ng/ml)
IL-10 production (ng/ml)
IL-12 production (ng/ml)
IL-23 production (ng/ml)
CXCL1 production (ng/ml)
CCL2 production (ng/ml)
CCL3 production (ng/ml)
CXCL10 production (ng/ml)
C-
WT
ΔcpsE
GBS III
GBS V
*

Figure 3. Impact **of MyD88 on cytokine release by DCs in response to GBS.** Control (CTRL) or MyD88$^{-/-}$ bmDCs were stimulated with GBS wild-type (WT) strains or their respective non-encapsulated (Δ*cpsE*) mutants (10^6 CFU/ml, initial MOI:1). After 2 h of bmDC-GBS infection, a bacteriostatic agent (chloramphenicol, 12 µg/ml) was added to the culture to prevent cell toxicity. Non-stimulated cells served as negative control (C-) for basal expression levels. Supernatants were harvested at 16 h of incubation and cytokine production quantified by ELISA. Data are expressed as mean ± SEM (in ng/ml) from eight independent experiments. * $P<0.05$, indicate statistically significant differences between WT strains and their respective non-encapsulated mutants.

With the exception of CXCL10, the production of cytokines and chemokines by bmDCs was either completely abrogated or markedly impaired in MyD88$^{-/-}$ bmDCs for all strains tested, independently of the CPS serotype (**Fig. 3**, $P<0.001$). It was demonstrated that LPS activation of MyD88$^{-/-}$ mouse macrophages results in impaired gene expression of various chemokines but not of CXCL10 compare to CTRL cells (52).

In contrast, MyD88 was partially involved in CXCL10 release by DCs exposed to the encapsulated pathogen *S. suis* (46).

TLR2 has a partial role in DC activation in response to GBS infection

The involvement of TLR2 in DC cytokine production following stimulation with GBS was also investigated using TLR2$^{-/-}$ bmDCs. We have previously demonstrated that the production of various cytokines by type III GBS-stimulated DCs is either partially-dependent or highly-dependent on bacterial internalization (28, 29). As TLR2 was not involved in GBS internalization, we firstly focused on those cytokines that seem to be mainly triggered upon GBS contact with a cell surface receptor (IL-6, TNF-α, CXCL1, CCL2 and CCL3 (28, 29)). As shown in **Fig. 4**, only the release of IL-6 and CXCL1 was partially reduced in TLR2$^{-/-}$ bmDCs infected by encapsulated GBS type III or type V strains ($P<0.05$). The non-encapsulated mutant of type III GBS showed a similar dependency on TLR2 than the WT strain. In contrast, IL-6 release by bmDCs stimulated with the non-encapsulated mutant of type V GBS was not affected by the absence of TLR2, yet this response was markedly MyD88-dependent (**Fig. 3**). It is thus likely that high levels of surface exposition of cell wall components (normally hidden by the type V CPS) are able to activate cells through multiple TLRs.

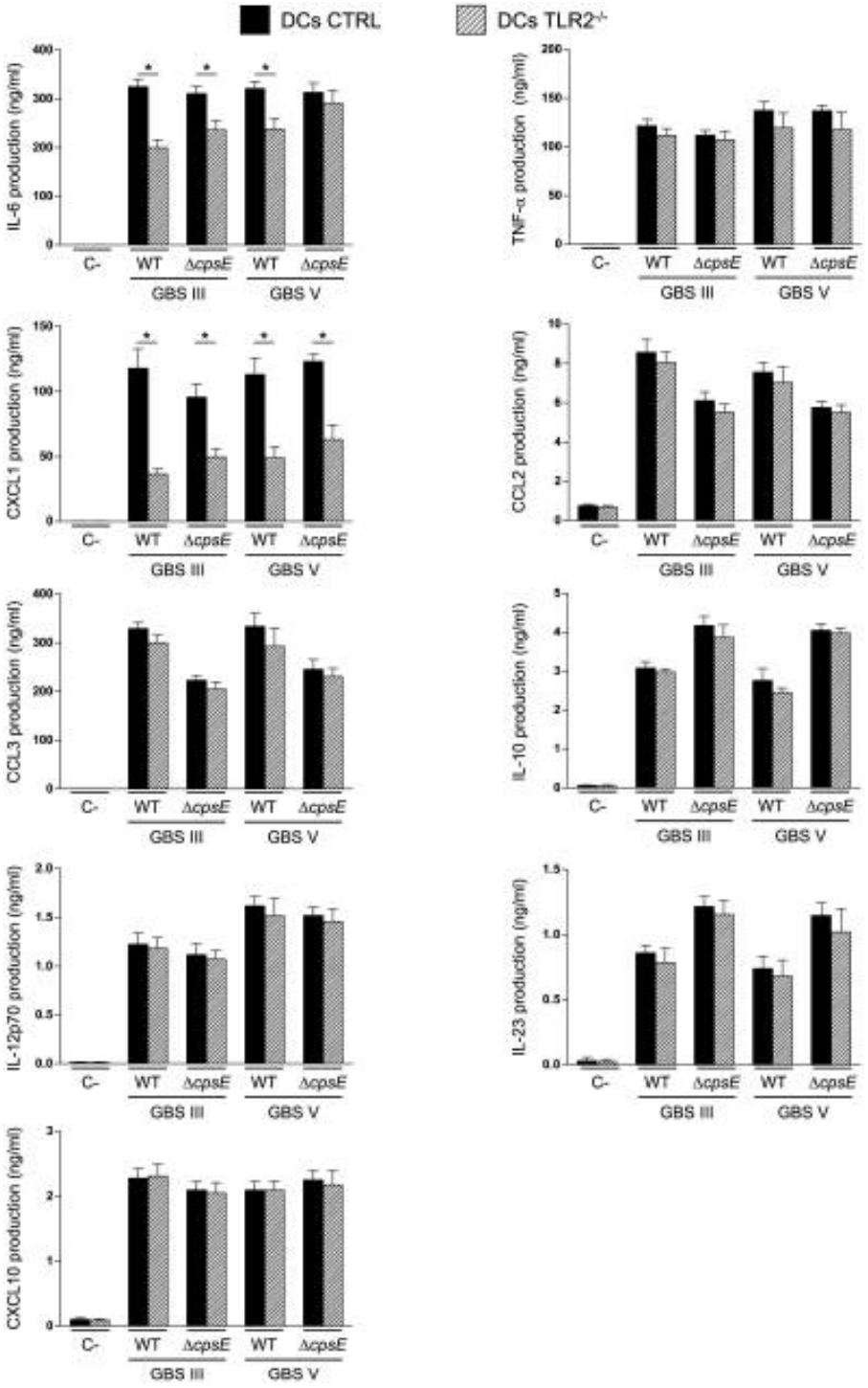

Figure 4. Effect of TLR2 on cytokine release by DCs in response to GBS. Control (CTRL) or TLR2-/- bmDCs were stimulated with GBS wild-type (WT) strains or their respective non-encapsulated (Δ*cpsE*) mutants (10^6 CFU/ml, initial MOI:1). After 2 h of bmDC-GBS infection, a bacteriostatic agent (chloramphenicol, 12 µg/ml) was added to the culture to prevent cell toxicity. Non-stimulated cells served as negative control (C-) for basal expression levels. Supernatants were harvested at 16 h of incubation and cytokine production quantified by ELISA. Data are expressed as mean ± SEM (in

ng/ml) from eight independent experiments. * $P<0.05$, indicate statistically significant differences between CTRL and TLR2-/- bmDCs.

The production of the cytokines IL-10, IL-12p70, IL-23 and CXCL10, known to be dependent on GBS internalization ((28) and unpublished data), was also evaluated and, as expected, no differences were observed in the capacity of $TLR2^{-/-}$ bmDCs to produce these cytokines compared to CTRL cells after either type III or type V GBS infection (**Fig. 4**).

Previous in vitro studies proposed that TLR2 is involved at a certain level in GBS-induced cell activation and cytokine production. Similarly to our data, the expression of a major fraction of genes in macrophages induced by whole heat killed-type III GBS does not significantly depend on TLR2, such as tnf, Ccl2, Ccl3, Il-12, and Cxcl10. On the other hand, and in agreement with features observed with DCs, induction of only few important molecules involved in host innate immunity, such as IL-6 and IL-1β, was impaired in the absence of TLR2 signaling in macrophages (18). Another study reported that activation of peritoneal macrophages by whole type III GBS is independent of TLR2 and TLR6, whereas a response to the secreted heat-labile soluble factor released by type III GBS was dependent on TLR2 (17). Furthermore, lipoteichoic acid from type III GBS is recognized as a TLR2/TLR6 ligand, but does not contribute significantly to GBS cell wall mediated macrophage activation (19). Albeit induction of IκB kinase activation (the kinase regulating NF-κB activation) but not that of JNK and p38 MAPKs in type V GBS-infected macrophages is blocked in the absence of MyD88, it is only partially inhibited in the absence of TLR2 (23). Finally, a study using human TLR2-transfected fibroblast cell line failed to demonstrate a role of TLR2 in cell interactions with heat killed-type III GBS compared to Listeria monocytogenes (21). In agreement with our results, Costa et al. have shown that TLR2 is not responsible for the release of TNF-α by type III GBS-infected bmDCs (24). In contrast, it has been demonstrated in vivo for both type III and type V GBS that TLR2/MyD88 mediates TNF-α and IL-6 production and contributes to bacterial clearance in a low-dose sepsis model, whereas it is detrimental in a high-dose model of septic shock due to an enhanced inflammatory response (22). In contrast, another study reported that $TLR2^{-/-}$ mice infected with type IV GBS show earlier and higher mortality rates and increased incidence and severity of disease than

control mice at all the infecting doses employed (53). Recently, Andrade et al. demonstrated that TLR2-induced IL-10 production is a key event in neonatal susceptibility to type III GBS sepsis (54). Thus, the role of TLR2 in GBS immuno-pathogenesis remains controversial and seems to depend on the infection model, the bacterial dose and/or the GBS serotype used. Yet, overall, TLR2 does not seem to play a major role in GBS activation of immune cells.

NOD2 does not seem to play a role in DC activation in response to GBS stimulation in vitro

We have previously reported that for type III GBS, the release of IL-10, IL-12p70, CXCL10 (28) and IL-23 is dependent on bacteria internalization by bmDCs (data not shown). Thus, we evaluated the role of NOD2, an important intracellular receptor, in the production of these cytokines by bmDCs upon infection with either type III or type V GBS. **Fig. 5** shows that $NOD2^{-/-}$ bmDCs produced the same amount of IL-10, IL-12p70, CXCL10 and IL-23 as CTRL bmDCs did when infected by either encapsulated type III or type V GBS, or by their respective non-encapsulated mutants. No differences were observed between CTRL bmDCs and $NOD2^{-/-}$ bmDCs in the release of other evaluated cytokines (**Fig. 5**; $P>0.05$).

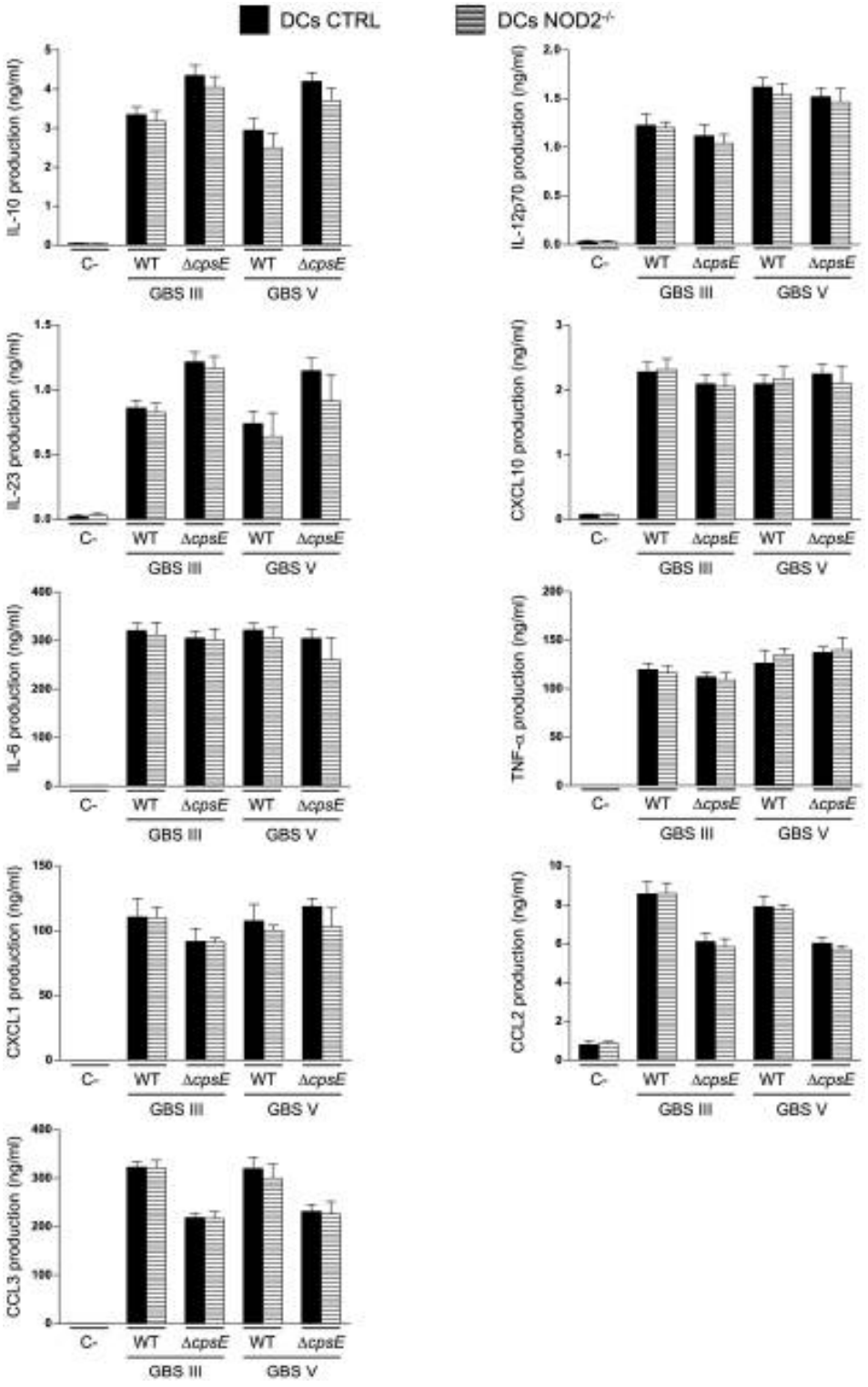

Figure 5. Effect of NOD2 on cytokine release by DCs in response to GBS. Control (CTRL) or NOD2$^{-/-}$ bmDCs were stimulated with GBS wild-type (WT) strains or their respective non-encapsulated (Δ*cpsE*) mutants (10^6 CFU/ml, initial MOI:1). After 2 h of bmDC-GBS infection, a bacteriostatic agent (chloramphenicol, 12 µg/ml) was added to the culture to prevent cell toxicity. Non-stimulated cells

served as negative control (C-) for basal expression levels. Supernatants were harvested at 16 h of incubation and cytokine production quantified by ELISA. Data are expressed as mean ± SEM (in ng/ml) from eight independent experiments.

In vitro studies performed with receptor-interacting protein 2 (RIP2) knockout macrophages, which lack NOD1 and NOD2 signaling, showed that both heat-killed and live type Ia GBS activate a potent IFN-β response similar to that of CTRL cells (26). Other study reported that NOD2 is dispensable for IκB kinase and MAPK activation in type V GBS-infected macrophages (23). In contrast, e*x vivo* analysis of total spleen cells from type III GBS-infected mice showed that the absence of NOD2 results in reduced production of inflammatory cytokines (27). Nevertheless, results presented here suggest that DCs do not contribute to this NOD2-dependent inflammatory response and that, instead, other immune cells would be involved. For example, it has been shown that NOD1 and NOD2 are necessary for optimal IFN-γ production by iNKT cells, as well as NK cells (55).

It has been shown that S. pneumoniae induces production of inflammatory cytokines in primary murine microglia, astrocytes and bone marrow-derived macrophages in a NOD2-dependent manner (56, 57). In the case of S. suis, Lecours et al. observed that the production of IL-23 and CXCL1 by infected-DCs is partially dependent on NOD2 (46). Our results seem to indicate that other intracellular receptors might be implicated in induction of cytokine release upon GBS internalization. Costa *et al.* reported that activation of the inflammasome, an inflammatory signaling complex, by type III GBS mediates *in vitro* production of IL-1β and IL-18, but not of TNF-α by DCs. Activation of the NLRP3 inflammasome requires GBS expression of β-hemolysin, an important virulence factor (24). In addition, TLR7 and TLR9 also recognize type III GBS nucleic acids in DC phagolysosomes after partial bacterial degradation, leading to IFN-β secretion (25). TLR9 sensing of type Ia GBS DNA was reported to be involved in the upregulation of TNF-α, IL-6 and IL-12 by mouse macrophages (58). In our study, the release of IL-10, IL-12p70 and IL-23 was shown to be MyD88-dependent. We speculate that TLR7 and TLR9 receptors are also implicated in the production of these cytokines by type III and type V GBS once internalized by DCs.

Conclusions

Progress has been achieved in recent years regarding our understanding of the complex interactions between GBS and DCs, especially in the context of type III GBS infection. However, due to limited information, it was unclear whether these interactions are similar or different among the diverse GBS serotypes. Here we demonstrated that type V GBS CPS partially impairs bacterial internalization, but does not improve bacterial intracellular survival, a pattern that slightly differs from that of type III GBS CPS. Interestingly, our results show that for both serotypes the TLR/MyD88 and NOD2 pathways do not modulate bacterial internalization. The TLR/MyD88 signaling cascade plays a major role in cytokine production by type III and type V GBS-infected DCs. Yet, TLR2 only partially contributes to DC activation by GBS of both serotypes. Upon internalization by DCs, both GBS types induce production of IL-10, IL-12p70 and IL-23 by a NOD2-independent but MyD88-dependent pathway, probably via an intracellular TLR. Notably, the production of the chemokine CXCL10 was shown to be MyD88/NOD2-independent. Induction of type I interferons and their regulatory pathways are probably involved in CXCL10 production. This pathway might involve TRIF activation instead of MyD88, but this remains a working hypothesis (59). Yet, a possible MyD88/TRIF-independent pathway for type I interferon production, as reported with GBS-infected macrophages cannot be ruled out (26). Altogether, our data support the hypothesis that GBS use complex TLR/MyD88-dependent in addition to TLR/NOD2-independent pathways to modulate host immune responses mediated by murine DCs.

In our model, the CPS only partially influences type III and type V GBS interactions with DCs. In fact CPS, as an antigen, only contributes to CCL2 and CCL3 production by DCs stimulated by either GBS serotype. In agreement with these results, these chemokines are the only ones produced by DCs in contact with highly purified type III or type V GBS CPS (32). In this study, CPS-mediated production of CCL3 was shown to be partially via TLR2 and MyD88-dependent pathways whereas CPS-induced CCL2 production involves TLR-independent mechanisms. Overall, no major differences were observed between type III and type V GBS in their interactions with DCs. However, further studies using multiple strains for each serotype are required to dissect the role of serotype in DC responses to GBS.

Albeit DCs and macrophages are immunologically distinct in their specific functions, surface receptors involved in GBS recognition by DCs seems to be similar to those reported in GBS interactions with macrophages. MyD88 signaling pathways are largely involved in GBS activation of both cell types, but TLR2 seems to play a minor role (17, 20, 60, 61). In contrast to surface receptors, intracellular sensing of GBS-derived molecules, including RNA and DNA, seems to occur in a cell-lineage specific manner, as reported by previous works (25, 26, 58, 61). All published studies addressing the interactions of GBS with DCs have been performed with murine-origin cells; it remains thus unclear how closely the activation of human DCs by GBS resembles the activation patterns observed with murine cells.

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ANNEXE; ARTICLE IX

Evaluation of the immunomodulatory properties of *Streptococcus suis* and group B *Streptococcus* capsular polysaccharides on the humoral response

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Publié dans: Pathogens. **2017**. 20;6(2). doi: 10.3390/pathogens6020016.

Détails sur le rôle du candidat dans la conception de l'article :
Je suis coauteur de l'article. J'ai construit les souches mutantes non encapsulées de GBS type V et *Streptococcus suis* sérotype 14 utilisées dans les expériences de cette articles. J'ai également génétiquement modifié une souche de GBS type III afin qu'elle exprime un lien modifié de l'acide sialique.

Abstract

Streptococcus suis and group B *Streptococcus* (GBS) are encapsulated streptococci causing septicemia and meningitis. Antibodies (Abs) against capsular polysaccharides (CPSs) have a crucial protective role, but the structure/composition of the CPS, including the presence of sialic acid, may interfere with the generation of anti-CPS Ab responses. We investigated the features of the CPS-specific Ab response directed against *S. suis* serotypes 2 and 14 and GBS serotypes III and V after infection or immunization with purified native or desialylated CPSs in mice. Whereas *S. suis*-infected mice developed a very low/undetectable CPS-specific IgM response, significant anti-CPS IgM titers were measured in GBS-infected animals (especially for type III GBS). No isotype switching was detected in *S. suis*- or GBS-infected mice. While the expression of sialic acid was essential for the immunogenicity of purified GBS type III CPS, this sugar was not responsible for the inability of purified *S. suis* types 2, 14 and GBS type V CPSs to induce a specific Ab response. Thus, other biochemical criteria unrelated to the presence of sialic acid may be responsible for the inaptitude of the host immune system to mount an effective response against certain *S. suis* and GBS CPS types.

Introduction

Streptococcus suis and *Streptococcus agalactiae* (also known as group B *Streptococcus* (GBS)) are two encapsulated bacteria that induce similar pathologies, including septicemia and meningitis, in animals and/or humans. *S. suis* is a major pig pathogen, responsible for important economic losses in the swine industry, as well as an emerging zoonotic pathogen in humans, responsible for deadly outbreaks in Asian countries. GBS is a leading cause of life-threatening invasive bacterial infections in neonates and pregnant women, as well as in the elderly and immunocompromised individuals. Among the 35 *S. suis* and 10 GBS serotypes identified, *S. suis* types 2 and 14 and GBS types III and V are the most virulent and frequently isolated [1,2].

For both pathogens, the capsular polysaccharide (CPS), which defines the serotype, is considered as a major virulence factor that protects the bacteria against host immune

responses [3-5]. However, the interplay of CPS with components of the innate immune system, including antigen-presenting cells (APCs), seems to differ radically between these two streptococci. Experiments using nonencapsulated mutants have shown that, in contrast to GBS CPS, *S. suis* CPS has a strong antiphagocytic effect and severely interferes with the activation and maturation of APCs [6-11]. Whereas the structures of GBS types III and V CPSs have been determined in the beginning of the 1990s [12,13], the structures of *S. suis* types 2 and 14 CPSs have only recently been elucidated [14,15]. The four CPSs are composed of glucose, galactose and *N*-acetylglucosamine and share structural features, being arranged into a repeating unit that contains a side chain terminated by sialic acid (*N*-acetylneuraminic acid). Remarkably, the presence of capsular sialic acid is a unique characteristic of *S. suis* and GBS among Gram-positive bacteria. Despite biochemical similarities, each CPS is made up of a unique arrangement of these sugars, conferring a distinct antigenicity. In addition, sialic acid forms an α-2,6 linkage with the adjacent galactose in *S. suis*, in contrast to the α-2,3 linkage found in GBS. Sialic acid of bacterial polysaccharides has been suggested to be involved in immune evasion mediated by molecular mimicry and by inhibition of complement activation, and this is possibly associated with the nature of sialic acid linkages [16-19]. Thus, the differential expression of sialic acid between *S. suis* and GBS might differentially modulate host immune responses.

The role of the humoral immunity and CPS-specific antibodies (Abs) in host defense against encapsulated bacteria is well established [20,21]. The efficacy of the protection of the different immunoglobulin (Ig) classes during bacterial infection is dependent on their affinity with cognate antigen and on their biological functions. IgG is particularly effective at mediating bacterial elimination by favoring bacterial opsonophagocytosis and/or by triggering the complement cascade directly at the surface of the pathogen [22,23]. In the case of *S. suis* and GBS, Abs directed against the CPS mediate protection in opsonophagocytosis assays and in vivo after passive transfer to animals before challenge [24-26]. Paradoxically, the cellular and molecular processes that lead to the development of CPS-specific Ab responses remain still elusive. By their inaptitude to recruit T cells during humoral responses, categorizing them as “thymo-independent” (TI) antigens, purified CPSs are usually less immunogenic than proteins [27,28]. Via their repeating epitopes, TI antigens are able to deliver strong and

sustained intracellular signaling through multivalent membrane Ig cross-linking at the surface of specific B cells, resulting in an efficient and robust cell proliferation [27,28]. However, the engagement of the B cell receptor by TI antigens is not sufficient to induce B cell activation, and a second signal is required, which may be provided by APCs via the release of B cell-activating factor of the tumor necrosis factor family (BAFF). In the absence of T cells, this cytokine promotes Ig class switching in naive B cells and their terminal differentiation into plasma cells [29]. Mice deficient in BAFF or its receptors display an abrogated IgG response specific to the synthetic TI model antigen, nitrophenol (NP)-Ficoll [30].

Ligands of Toll-like receptors (TLRs) may also potentiate TI Ab responses. The engagement of TLR4 by LPS stimulates expression and production of BAFF by APCs, including dendritic cells (DCs) [29,31]. In addition, TLR9 agonists CpG oligodeoxynucleotides (ODNs) induce an increased expression of BAFF receptors by B cells [32]. However, the effectiveness of TLR ligands as adjuvants seems to depend on the nature of the TI antigen. For example, in mice, whereas CpG ODNs significantly increase the IgM and IgG responses to NP-Ficoll [32,33] and to purified *Streptococcus pneumoniae* type 3 CPS (PS3) [34], it does not heighten the immunogenicity of purified *S. pneumoniae* types 6B, 19F and 23F CPSs [33].

Some studies suggest that CPSs may also have intrinsic immunosuppressive properties. For example, *Neisseria meningitidis* group C CPS or certain types of pneumococcal CPSs induce hyporesponsiveness after immunization [35,36]. These bacterial CPSs were shown in vitro to inhibit the maturation and the pro-inflammatory activities of human macrophages and/or DCs and polarize immune responses toward a regulatory profile [37,38]. We have previously shown that purified *S. suis* and GBS CPSs partially inhibit BAFF expression in murine DCs [39]. However, it is unclear how these properties of CPSs influence the generation of the humoral immunity.

The goal of this study was to evaluate the influence of the CPS biochemistry on the development of the humoral response by comparing the features of CPS-specific Ab responses

against *S. suis* types 2, 14 and GBS types III and V in mice. We first determined the characteristics of this response after a clinical infection with live virulent strains and after immunization with purified CPSs. We then investigated the in vitro interactions of each purified CPS with murine B cells and soluble factors involved in TI Ab responses, including CpG ODNs and BAFF. Finally, the influence of sialic acid was analyzed using chemically-desialylated CPSs.

Results

Distinct Features of the CPS-Specific Ab Response between Mice Infected with Live *S. suis* and GBS

In order to compare the features of the humoral immunity against *S. suis* and GBS CPSs, we first infected mice with live virulent strains of *S. suis* types 2 or 14 or GBS types III or V and evaluated the kinetics, magnitude and isotype profile of the induced anti-CPS Ab response. The protein-specific Ab response was also measured in parallel for each bacterial strain. Bacteremia was detected in all surviving mice at 12-h post-infection, ranging from ~10^4 to 10^7 CFU/mL for *S. suis* types 2 and 14, from 10^2 to 10^6 CFU/mL for GBS type III and from 10^2 to 5×10^3 CFU/mL for GBS type V, in accordance with previous studies [5,40,41] (Figure S1).

Mice infected with *S. suis* types 2 and 14 developed a significant anti-protein Ab response that was mainly composed of IgG, with an almost 300-fold increase in titers versus non-infected mice (Figure 1A,B). In contrast to the protein response, either a weak anti-CPS IgM response was observed from Day 14 to Day 21 post-infection in *S. suis* type 2-infected mice; or an almost complete absence of significant anti-CPS titers was noticed in *S. suis* type 14-infected animals (Figure 1A,B). There was a positive correlation between the anti-protein Ab titers and the bacteremia in mice infected with *S. suis* type 2 or 14, but not between the anti-CPS titers and the bacteremia in *S. suis* type 2-infected mice [41] (data not shown).

In opposition to *S. suis*-infected mice, GBS-infected animals presented a significant CPS-specific Ab response as soon as Day 7, which remained stable until Day 21 (Figure 1C,D).

However, in GBS type V-infected mice, the magnitude was also relatively low, with an eight-fold increase in IgM titers compared to control mice. On the other hand, GBS type III-infected mice showed a 20-fold increase in anti-CPS IgM titers; though, no IgG anti-CPS titers were detected in GBS-infected mice (Figure 1C,D). Surprisingly, even if some GBS type III- or GBS type V-infected mice exhibited a significant protein-specific Ab response, this response was globally low. It should be noted that higher GBS bacterial doses were lethal early after infection and thus could not be evaluated. No correlation was found between the anti-CPS Ab titers and the bacteremia in GBS-infected mice. The low levels of Abs and/or blood bacteremia might preclude a positive correlation, which might vary upon the infection model.

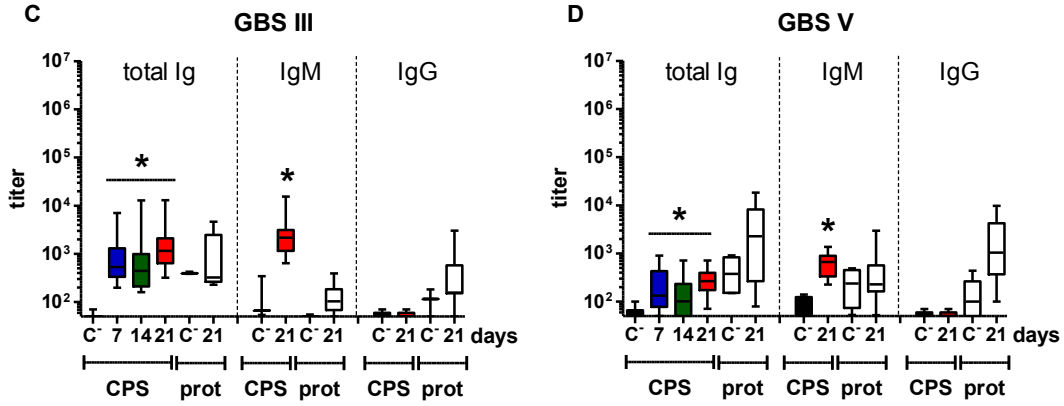

Figure 1. Titration of capsular polysaccharide (CPS)-specific antibodies in mice after infection with *S. suis* serotype 2, *S. suis* serotype 14, group B *Streptococcus* (GBS) serotype III or GBS serotype V. Mice were infected with 2×10^7 CFU of live *S. suis* serotype 2 strain P1/7 ($n = 60$) (A); 5×10^6 CFU of live *S. suis* serotype 14 strain DAN13730 ($n = 40$) (B); 2×10^6 CFU of live GBS serotype III strain COH-1 ($n = 40$) (C); or 10^4 CFU of live GBS serotype V strain CJB111 ($n = 40$) (D). Total Ig (IgG plus IgM) anti-CPS titers were determined by ELISA on Days 7 (in blue), 14 (in green) and 21 (in red) in surviving mice. IgM and IgG anti-CPS titers were determined on Day 21 (in red). For comparative purpose, total Ig (IgG plus IgM), IgM and IgG anti-protein ('prot') titers were also determined on Day 21. Data are presented in a box-and-whiskers diagram with the ends of whiskers representing the minimum and the maximum value. "C^-" represents a pool of control mice ($n = 3$) injected with vehicle solution, whose titers were evaluated on Days 7, 14 and 21. * Statistically significant difference ($p < 0.05$) in comparison to the respective C^- group.

Features of the CPS-Specific Ab Response in Mice Immunized with Purified *S. suis* and GBS CPSs

A live bacterium contains numerous protein and polysaccharide antigens that are secreted and/or intimately co-expressed within an organized particulate structure owning multiple adjuvanting moieties. This may confer unique immunogenic properties to the CPS expressed in the context of an intact bacterium [21]. In order to directly evaluate the influence of the CPS biochemistry on the development of the humoral immunity against *S. suis* and GBS CPSs, we then immunized mice with purified *S. suis* type 2 or 14 or GBS type III or V CPS and compared the features of the induced primary anti-CPS Ab response (Figure 2).

Like with live bacteria, the specific Ab response in mice immunized with *S. suis* type 14 CPS remained similar to that of the negative control group throughout the experiment (Figure 2B). Unlike mice infected with live *S. suis* type 2 or GBS type V, anti-CPS Ab titers were no longer measured in mice immunized with the CPS purified from these two pathogens (Figure 2A,D). In contrast, we observed a significant response in mice immunized with purified CPS from GBS type III, with similar amplitude and kinetics to what was monitored after infection with the bacteria (Figure 2C). This response was composed of IgM, and no IgG was detected (Figure S2). In accordance with the inability of purified TI antigens to generate an amplified reaction subsequent to a second administration [42], no significant difference in the kinetics, magnitude or isotype profile was observed between primary and secondary specific Ab responses against each of the four CPSs (Figure S3). Therefore, whereas the presence of subcapsular and/or secreted bacterial components may be required for the generation of *S. suis* type 2 and GBS type V CPS-specific Abs in mice, GBS type III CPS possesses intrinsic immunogenic properties. *S. suis* type 14 CPS has no immunogenic potential, even when associated with the bacterial surface.

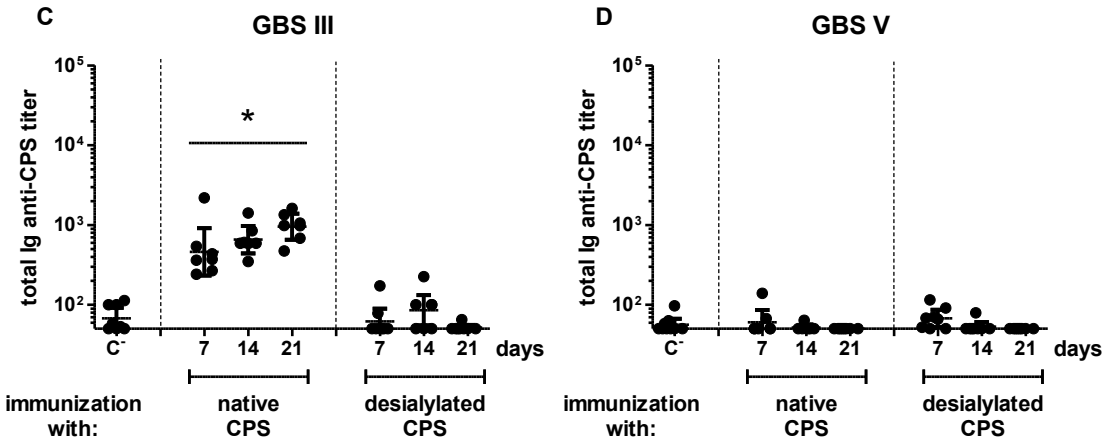

Figure 2. Titration of CPS-specific antibodies in mice after immunization with purified native or desialylated *S. suis* serotype 2, *S. suis* serotype 14, GBS serotype III or GBS serotype V CPS. Mice (n = 8) were immunized with 2 µg of purified native or desialylated *S. suis* serotype 2 (A); *S. suis* serotype 14 (B); GBS serotype III (C); or GBS serotype V (D) CPS emulsified with STIMUNE®. Total Ig (IgG plus IgM) anti-native CPS titers were determined by ELISA on Days 7, 14 and 21. "C^-" represents a pool of control mice (n = 3) injected with STIMUNE® only, whose titers were evaluated on Days 7, 14 and 21. Data from individual mice are presented, including the geometric mean with 95% confidence interval. * Statistically significant difference ($p < 0.05$) in comparison to the C^- group.

Influence of Sialic Acid on the Features of the CPS-Specific Ab Response in Mice Immunized with Purified *S. suis* and GBS CPSs

The expression of sialic acid by pathogens interferes with the host immunity by preventing complement activation on microbial surfaces [16,17], as well as by binding to inhibitory receptors expressed by immune cells [18,19]. However, the consequences of sialic acid expression on the development of humoral responses remain poorly explored. As the deletion of genes involved in sialic acid synthesis results in considerable or complete loss of CPS expression by *S. suis* and GBS [5,43,44], the influence of this sugar has been evaluated by using purified, chemically-desialylated CPSs. As shown in Figure 2 and Figure S3, no significant difference in the CPS-specific Ab response was observed between mice immunized with native and desialylated *S. suis* type 2, *S. suis* type 14 or GBS type V CPS. In contrast, a significant decrease in GBS type III CPS-specific Ab response was detected in mice immunized with desialylated CPS, with Ab titers returning to basal levels of the placebo group (Figure 2C and Figure S3C). This is in accordance with previous studies suggesting that sialic

acid exerts a conformational control of the helical structure of the immunodominant epitope of this CPS [45]. To test this hypothesis and further evaluate the impact of the sialic acid α-2,3 linkage vs. α-2,6 linkage, we generated α-2,6 sialylated GBS type III CPS (see Appendix A). Compared to native (α-2,3) CPS, the (α-2,6) CPS completely lost its immunogenic capacity as a non-significant Ab response against the native CPS could be detected in mice immunized with this modified polysaccharide (Figure A1, left panel). Nevertheless, a low albeit significant Ab response against the α-2,6 modified form itself was observed, suggesting the generation of a new epitope with no cross-reaction with the native form (Figure A1, right panel). Therefore, whereas the presence of sialic acid may not exert a major modulatory effect on the poor ability of purified *S. suis* types 2, 14 and GBS type V CPSs to induce a specific Ab response in mice, the expression of this sugar (in its α-2,3 linkage) is essential for the immunogenicity of GBS type III CPS.

In Vivo Effect of Exogenous TLR Agonists on *S. suis* Type 2 CPS-Specific Ab Response

In comparison with *S. suis* type 2 CPS expressed in the context of an intact bacteria, the complete loss in the immunogenicity of the same antigen administrated under a purified form suggests that bacterial non-capsular factors could potentiate the development of CPS-specific humoral response. We thus evaluated if an exogenous TLR agonist, expected to mimic some of the co-signals provided by whole bacteria, could modulate the magnitude of the Ab response directed to purified *S. suis* type 2 CPS (selected as a model CPS for the subsequent study). We focused our work on CpG ODNs because previous studies have demonstrated that these TLR9 agonists amplified the humoral reaction against purified bacterial CPS, provided that they were administrated a few days after immunization with CPS [34,46]. Nevertheless, following the same experimental protocol, no improvement of the *S. suis* type 2 CPS-specific serum Ab response was obtained when CpG ODNs were injected into mice, and Ab titers remained undetectable from Day 7 to Day 21 (Figure 3A).

This was in sharp opposition with the significant adjuvant effect observed on total Ig (IgG plus IgM) PS3-specific serum Ab response during the same period (Figure 3A). Like in previous reports [34,46], CpG ODNs promoted a rise of both IgM and IgG anti-PS3 titers

(Figure S4). The inability of CpG ODNs to potentiate *S. suis* type 2 CPS-specific Ab response may not be related to the expression of sialic acid, as a similar result was obtained in mice immunized with desialylated CPS plus CpG ODNs (Figure 3A). In accordance with the results obtained with serum Ab titers, the injection of CpG ODNs significantly increased the number of splenic anti-PS3 Ab-secreting cells (ASCs), whereas specific ASCs were not significantly detected in the spleen of mice immunized with native or desialylated *S. suis* type 2 CPS, whether mice received CpG ODNs or not (Figure 3B,C).

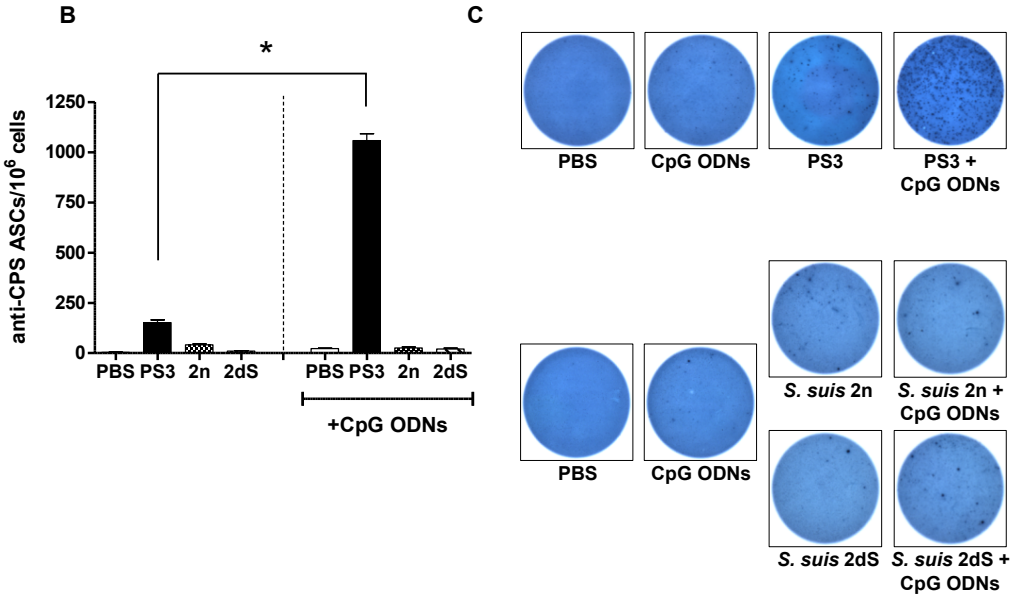

Figure 3. Adjuvant effect of CpG oligodeoxynucleotides (ODNs) on the CPS-specific humoral response in mice immunized with purified native or desialylated *S. suis* serotype 2 CPS or purified *S. pneumoniae* serotype 3 CPS (PS3). (A) In a first set of experiments, mice (n = 8) were immunized with 2 µg of purified native (n) or desialylated (dS) *S. suis* serotype 2 CPS (CPS *S. suis*) or PS3 in PBS on Day 0 and 80 µg of CpG ODNs two days after. The control group (n = 8) received CPS or PS3 on Day 0 and PBS two days later. The placebo group (n = 3) received PBS or CpG ODNs only. Total Ig (IgG plus IgM) anti-native *S. suis* type 2 CPS or anti-PS3 titers were determined by ELISA on Days 7, 14 and 21. Data are presented in a box-and-whiskers diagram with the ends of whiskers representing the minimum and the maximum value. (B) In a second set of experiments, mice (n = 5) were immunized as described in (A), but splenocytes were collected on Day 5 after immunization, and anti-CPS antibody-secreting cells (ASCs) were enumerated by ELISpot as described in the Materials and Methods section. Data are expressed as arithmetic means with SEM. (C) Visualization of PS3 (top) or native *S. suis* type 2 CPS (bottom) specific ASCs in ELISpot wells from splenocytes of mice obtained in (B). * $p < 0.05$ between "PS3" and "PS3 + CpG ODNs" groups.

In Vitro Study of the Immunomodulatory Effect of Purified *S. suis* and GBS CPSs

In order to get a better understanding of the cellular and molecular mechanisms responsible for the poor immunogenicity of *S. suis* types 2 and 14 and GBS type V CPSs, as well as the relatively higher ability of GBS type III CPS to induce a specific Ab response, we compared the in vitro interactions of each purified CPS with naive B cells isolated from mouse spleen, a central organ in the implementation of Ab reaction against TI antigens [47,48]. In particular, we evaluated the immunomodulatory effect of CPSs on the capacity of B cells to secrete Ig in response to soluble factors critically involved in the generation of TI humoral immunity. We first assessed the influence of CPS on the ability of BAFF to promote Ig secretion by B cells. Stimulation with BAFF along with IL-4 was required for Ig production, and cells incubated with media or each CPS alone secreted very low levels of IgM or IgG (Figure 4, left panels).

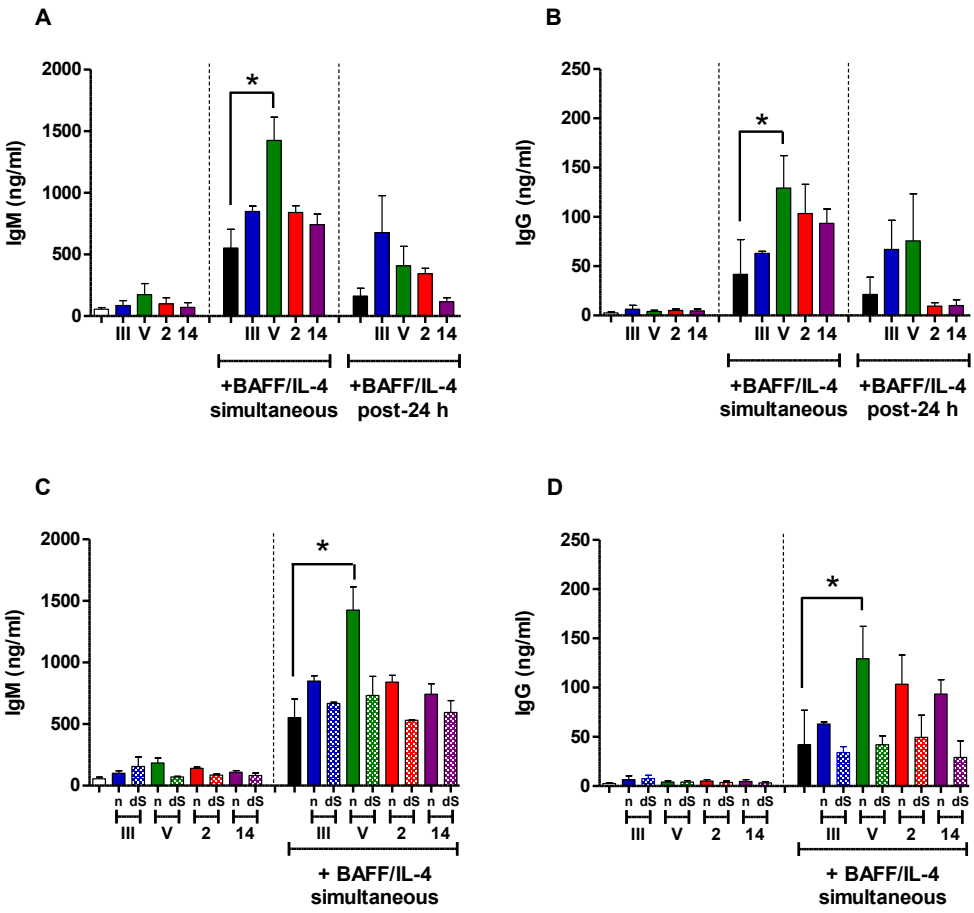

Figure 4. In vitro immunomodulatory effect of purified native or desialylated *S. suis* serotype 2, *S. suis* serotype 14, GBS serotype III or GBS serotype V CPS on BAFF/IL-4-induced Ig secretion by naive B cells. (A,B) Mouse splenic B cells (10^6 cells/mL) were incubated with purified native *S. suis* serotype 2, *S. suis* serotype 14, GBS serotype III or GBS serotype V CPS (each at 20 µg/mL) simultaneously with (central panel) or 24 h before the addition of (right panel) BAFF (1 µg/mL) along with IL-4 (50 ng/mL); (C,D) in order to evaluate the influence of sialic acid, cells were also incubated with desialylated (dS) *S. suis* serotype 2, *S. suis* serotype 14, GBS serotype III or GBS serotype V CPS in parallel to cells incubated with the respective native CPS (n) as described in (A,B). After seven days of incubation, supernatants were collected, and total IgM (A,C) and IgG (B,D) was quantified by ELISA. Cells stimulated with BAFF/IL-4 alone (black bars) served as a positive control. In the left panel, cells stimulated with medium (white bars) or each purified CPS alone are represented as an indication of the basal Ig secretion level of cells in absence of BAFF/IL-4 stimulation. Data are expressed as arithmetic means with the SEM of three (central and right panels) or four (left panels) experiments. * $p < 0.05$.

There was an overall tendency to an increase of the Ig production by B cells incubated with the CPS plus BAFF/IL-4 in comparison with cells incubated with BAFF/IL-4 only,

whether CPS was added 24 h before or simultaneously to BAFF/IL-4, and this seemed to be related to the presence of sialic acid. We then evaluated the effect of *S. suis* and GBS CPSs on the Ig secretion by B cells induced by CpG ODNs. The addition of the different CPSs (either native or desialylated) did not influence the ability of CpG ODNs to bring about IgM or IgG production by B cells, and a similar conclusion was obtained when CPS was added 24 h before or simultaneously to CpG ODNs (Figure 5 and Figure S5).

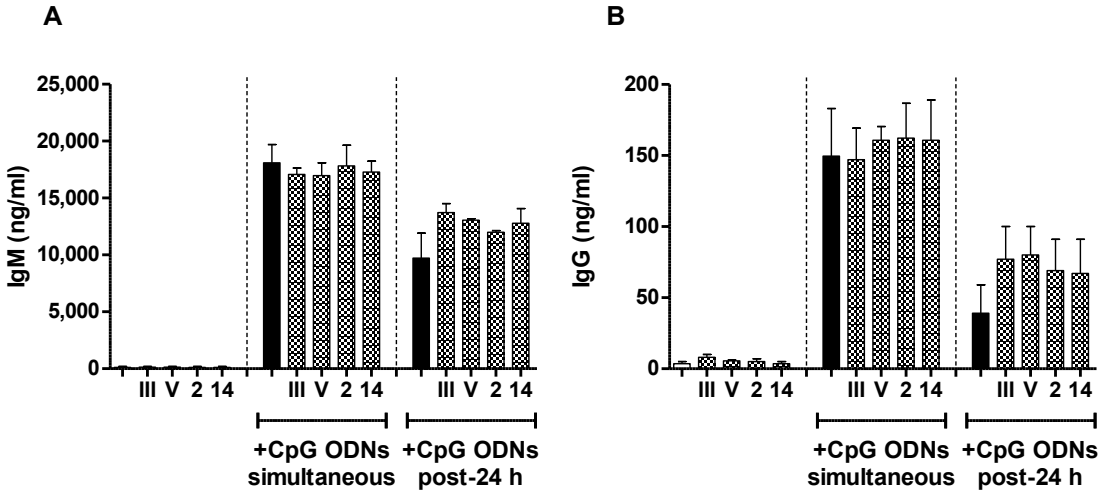

Figure 5. In vitro immunomodulatory effect of purified native *S. suis* serotype 2, *S. suis* serotype 14, GBS serotype III or GBS serotype V CPS on the Ig secretion by naive B cells induced by CpG ODNs. Mouse splenic B cells (10^6 cells/mL) were incubated with purified native *S. suis* serotype 2, *S. suis* serotype 14, GBS serotype III or GBS serotype V CPS (each at 20 µg/mL) simultaneously with (central panel) or 24 h before the addition of (right panel) CpG ODNs (1 µg/mL). After seven days of incubation, supernatants were collected and total IgM (A) and IgG (B) were quantified by ELISA. Cells stimulated with CpG ODNs alone (black bars) served as the positive control. In the left panel, cells stimulated with medium (white bars) or each purified CPS alone are represented as an indication of the basal Ig secretion level of cells in absence of stimulation by CpG ODNs. Data are expressed as arithmetic means with the SEM of three (central and right panels) or four (left panel) experiments.

Therefore, in our hands, the poor immunogenicity of *S. suis* CPSs and GBS type V CPS could not be explained by the induction of an immunosuppressive state of B cells, making them unreactive to the stimulation by BAFF/IL-4 or CpG ODNs.

Study of the Immunomodulatory Effect of Purified *S. suis* and GBS CPSs on a T Cell-Dependent Response

In the light of the reported immunosuppressive effect of encapsulated bacteria on the generation of humoral immunity specific to heterologous proteins [49], as well as the ability of purified microbial polysaccharides to interfere with the presentation of T cell-dependent (TD) antigens by APCs [50], we assessed if purified *S. suis* and GBS CPSs could prevent the development of ovalbumin (OVA)-specific Ab response in mice. We chose this TD antigen model because bacterial CPSs have been previously described to impede the activation of OVA-specific T cells [51]. As shown in Figure 6, the co-injection of OVA with *S. suis* type 2 or 14 or GBS type III or V CPS did not hamper the generation of primary OVA-specific Ab response.

Except for a slight inhibitory effect of *S. suis* type 14 CPS, no major influence of *S. suis* or GBS CPS was noticed on the generation of the memory OVA-specific Ab response. Similar results were obtained regardless of the concentration of CPS (2 or 20 μg) and the route of immunization (s.c. or i.p.) (data not shown).

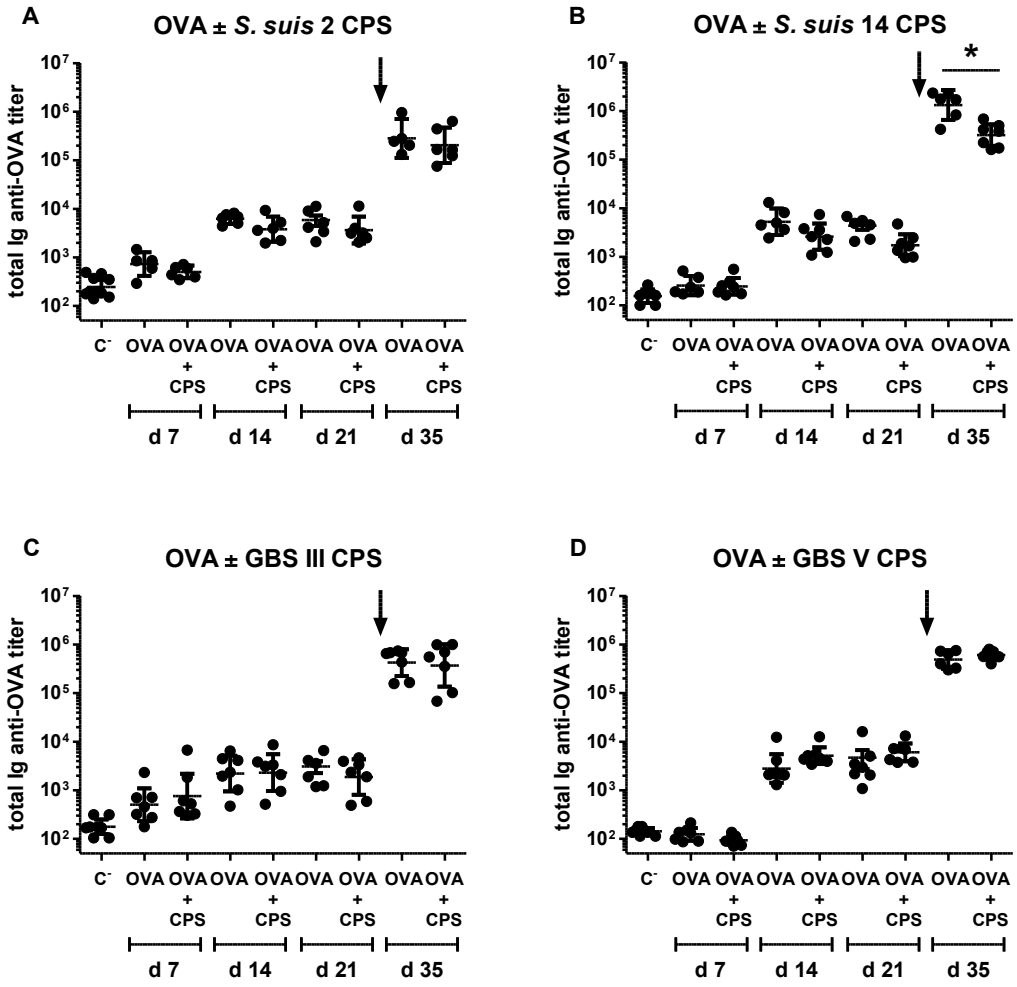

Figure 6. In vivo immunomodulatory effect of purified native *S. suis* serotype 2, *S. suis* serotype 14, GBS serotype III or GBS serotype V CPS on ovalbumin (OVA)-specific antibody response. Mice (n = 8) were co-immunized intraperitoneally with 10 µg of OVA and 2 µg of purified native *S. suis* serotype 2 (A), *S. suis* serotype 14 (B), GBS serotype III (C) or GBS serotype V (D) CPS in PBS on Days 0 and 21. Control group (n = 8) received OVA only on Days 0 and 21. Total Ig (IgG plus IgM) anti-OVA titers were determined by ELISA on Days 7, 14, 21 and 35. "C⁻" represents a pool of placebo mice (n = 3) injected with PBS, whose titers were evaluated on Days 7, 14, 21 and 35. Data from individual mice are presented, including the geometric mean with 95% confidence interval. An arrow indicates secondary immunization. * $p < 0.05$.

Discussion

The humoral response is the main mechanism of host adaptive immunity in the fight against infections with extracellular pathogens. Via their biological functions of opsonophagocytosis, CPS-specific Abs allow clearance of bacteria by the immune system. This is the rationale for the development of vaccines against *S. pneumoniae*, *N. meningitidis* and *Haemophilus influenzae* infections in humans [20,21]. Despite being an important pig pathogen and an emerging threat to human health, no efficient vaccine against *S. suis* is currently available [52]. Whole-cell killed bacterins used in the field provide limited protection, and experimental live-attenuated vaccines have been tried with contradictory results and the inherent risk of zoonosis. Moreover, the high genotypic and phenotypic variations among *S. suis* strains of different geographical origins may preclude the use of protein subunit vaccines [52]. Concerning GBS, despite the fact that protein-based vaccines confer potent defense in clinical trials, they generally do not provide broad-coverage protection [53]. For both *S. suis* and GBS, the CPS represents a target of choice, not only because CPS-specific Abs display a good protective potential in experimental studies [24-26], but also because a vaccine composed of CPSs purified from several serotypes would be the key to a universal vaccine. However, the design of CPS-based vaccines is an area of research only recently explored for *S. suis* [54], and the efficacy of in-trial GBS vaccines depends on the serotype of the CPS included in the preparations [26,55]. 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 than IgG. Yet, the mechanisms for the generation of *S. suis* and GBS CPS-specific Ab responses, as well as the influence of intrinsic immunomodulatory properties of CPS on its immunogenicity have been poorly explored. Our work is the first to compare, within the same study, the impact of the composition and/or structure of *S. suis* types 2, 14 and GBS types III and V CPSs on the generation of humoral responses, including the influence of the presence of sialic acid.

Bacterial CPSs are classically encountered by the host immune system covalently attached to the underlying subcapsular domain. We thus initially compared the anti-CPS Ab response subsequent to a clinical infection with live virulent *S. suis* type 2 or 14 or GBS type

III or V in mice. Our results showed that CPS-specific Ab responses to whole GBS or *S. suis* exhibit typical features of a TI reaction in spite of CPS association with the bacterial surface. It is interesting to note that similar findings, in terms of poor anti-CPS antibody response, were observed with other *S. suis* serotype 2 strains [41,56], suggesting that the characteristics of the anti-CPS antibody response do not seem to be influenced by the composition and/or architecture of the bacterial subcapsular domain, at least in the case of *S. suis*. Regardless of this common TI nature, infection with GBS type III resulted in a significant production of anti-CPS Abs, whereas a weak or negative humoral anti-CPS response was observed after infection with other bacterial types, especially for *S. suis*. The dissimilar CPS-specific Ab responses subsequent to infection with *S. suis* and GBS could be attributed to differences in the kinetics of bacterial multiplication, dissemination and survival in the host. In this regard, the total anti-protein response was also dissimilar between these two bacterial species. Previous studies demonstrated that the expression of CPS differently modulated the interactions of whole *S. suis* and GBS with the host innate immunity. Experiments using nonencapsulated mutant strains showed that *S. suis* types 2 and 14 CPSs crucially protected bacteria from phagocytosis by APCs, including DCs and macrophages [5-8,11]. Remarkably, *S. suis* type 2 CPS did not act simply as an inert physical barrier against phagocytes, but actively down-modulated signaling pathways involved in phagocytosis [8,57]. In contrast to *S. suis*, GBS types III and V were efficiently internalized by APCs [6-11]. Therefore, the different interplay of *S. suis* and GBS with innate immune cells during the course of infection may promote distinct adaptive immune reactions and may explain the disparate CPS- and protein-specific humoral response that were observed between the two pathogens. The different and globally-weak CPS-specific Ab responses subsequent to infection with *S. suis* and GBS could also be attributed to the immunomodulatory influence of secreted factors and/or non-capsular components expressed within the bacteria. In contrast to *S. suis* and GBS, it was reported that immunization with intact heat-killed *S. pneumoniae* capsular type 14 [58] or *N. meningitidis* serogroup C [59] induced significant IgM and IgG anti-CPS responses. The authors suggested that the nature of the in vivo anti-CPS response was markedly influenced by the composition and/or architecture of the bacterial subcapsular domain [60]. Using inactivated GBS type III immunization, not only a relatively high anti-CPS IgM response, but also modest IgG titers were observed [60]. This observation adds another level of complexity to the analysis of the features of the anti-

CPS responses against whole bacteria, as live organisms might behave differently from inactivated ones. Therefore, the distinct CPS-specific Ab responses that we have observed in our experimental infections with live bacteria, such as the IgM-restricted isotype profile, might also be related to the influence of antigens that are selectively produced in vivo.

Several studies have suggested that the anti-CPS responses to intact bacteria versus isolated CPS are distinct [21]. For example, whereas purified *S. pneumoniae* type 14 CPS behaved as a TI antigen, its expression at the surface of intact bacteria markedly increased its immunogenicity in mice [58,61]. This included a newly-acquired capacity to recruit T cell help in the development of humoral response, displayed by high titers of CPS-specific IgG. In the present study, the anti-CPS response against *S. suis* type 2 and GBS type V (but not that of GBS type III) was absolutely dependent on CPS expression on the bacterial surface. In the case of purified *S. suis* types 2 and 14 CPSs, the complete lack of immunogenicity as a soluble antigen was observed at different doses (ranging from 1 to 25 μg) and despite the addition of different adjuvants [54] (unpublished observations). Of note, the TLR4 agonist monophosphoryl lipid A did not exert any adjuvant effect on *S. suis* type 2 CPS-specific Ab response in mice (Figure S6), despite the fact that this ligand has the capacity to restore the immunogenicity of a synthetic sialylated TI antigen [62]. Moreover, and in contrast to PS3 [34,46], the TLR agonists CpG ODNs were also unable to rescue soluble *S. suis* type 2 CPS immunogenicity. Co-immunization with purified PS3 and CpG ODNs enhanced the CPS-specific Ab response in mice, with similar efficiency and longevity as a PS3-tetanus toxoid conjugate vaccine [34,46]. It was demonstrated that CpG ODNs exerted their adjuvant effect directly on TI-specific B cell clones by promoting survival, proliferation and plasma cell differentiation [46]. Similar to our results, co-injection of mice with CpG ODNs and purified *H. influenzae* type b or *N. meningitidis* group C CPS did not improve the specific humoral response [63,64]. Thus, the Ab response to bacterial CPSs cannot be generalized as important inter-species differences exist. Furthermore, the immunogenicity of the CPS is affected by its presentation form (associated with the bacterial wall or soluble); though this effect also depends on the CPS type.

Besides secreted and/or closely co-expressed non-capsular antigens by the bacteria, intrinsic properties of the CPS itself may direct the development of CPS-specific Ab responses. One element that may play a crucial role is sialic acid. This sugar is widely expressed at terminal positions of glycoconjugates exposed at the surface of most mammalian leucocytes, and by interacting with sialic-acid binding lectins (Siglecs) expressed on the same cell (*cis* interaction), it maintains a constitutive inhibitory tone of immune cells [18,65-68]. Remarkably, sialylated bacteria can exploit these receptors to dampen immune responses [18,19]. However, our study suggests that sialic acid does not significantly modulate the generation of Ab response specific to *S. suis* and GBS CPSs. Furthermore, the presence of sialic acid in its native α-2,3 linkage is absolutely required for GBS type III CPS immunogenicity. In contrast to our results, chemical alteration of sialic acid of purified *N. meningitidis* group B CPS greatly improved immunogenicity of the CPS, including IgG isotype switching in mice [69]. A CPS-specific IgM-to-IgG isotype switching was also observed in macaques immunized with purified desialylated GBS type V CPS in comparison with animals receiving the native CPS [55]. However, a TD form of CPS (CPS conjugated to a carrier protein) was used in both studies, and thus, the participation of T cells in the development of the humoral response may explain the divergent conclusions with the present work. In this regard, modifications of sialic acid of unconjugated *N. meningitidis* group B CPS had no influence on its immunogenicity [69]. Variations in the composition of the repeating units unconnected to the presence of sialic acid, in the molecular weight of the CPS, as well as in the spacing, the rigidity and the multivalence of the epitopes of the CPS may then account for the different immunogenic properties of purified GBS type III versus *S. suis* types 2, 14 and GBS type V CPSs. It is interesting to note that the chemical modification of purified *S. suis* type 2 [54] and GBS type V CPSs [55] by a covalent conjugation to a carrier protein conferred to these molecules the ability to trigger a CPS-specific Ab response, indicating that the non-immunogenicity of these purified CPSs is not due to the absence of CPS-specific B cells in the repertoire of the host.

Purified B cell subsets preferentially involved in TI responses are particularly reactive to in vitro stimulation by TLR ligands [70]. In addition, these molecules induced BAFF production by innate immune cells [29,31], promoted the expression of BAFF receptors by B

cells [32] and the co-engagement of TLRs and BAFF receptors synergized to improve B cell activation in the absence of T cell help [71]. As such, some evidence suggests that the poor immunogenicity of bacterial CPSs is related to their suppressive action on BAFF and its receptors [64]. Whereas we have previously demonstrated that purified *S. suis* types 2, 14 and GBS types III and V CPSs partially impaired in vitro BAFF expression by murine DCs [39], in the present study, we were unable to see a down-modulatory effect of these CPSs on the ability of exogenous BAFF to induce in vitro Ig secretion by murine B cells. Furthermore, we did not observe either an immunosuppressive or immunomodulatory effect of purified (either native or desialylated) *S. suis* CPS or GBS CPS on the Ig secretion by B cells mediated by CpG ODNs in our in vitro culture system. If these CPSs exhibit immunosuppressive effects, they may thus exert their action preferentially towards APCs and not B cells. Contrary to our results, in addition to blunting the production of BAFF by DCs, purified *N. meningitidis* group C CPS partially inhibited the in vitro response of murine B cells to BAFF stimulation [64]. The strong immunosuppressive effect of the *N. meningitidis* group C CPS can be explained by the fact that it is an α-2,9 linked homopolymer of sialic acid. The highest degree of presentation of sialic acid molecules by *N. meningitidis* group C CPS might result in an interaction of higher affinity with host Siglecs than *S. suis* and GBS CPSs. Variations in experimental conditions could also account for the observed differences, including the mouse strain origin and the subsets of B cells used. Indeed, our in vitro B cell culture model did not include all of the B cell subsets thought to be involved in TI Ab responses. In particular, B1 B cells, an important cellular population in polysaccharide-specific Ab responses [42,48], were counter-selected during the protocol of purification (these cells express the surface marker CD43), and we have focused our work on the interactions of *S. suis* and GBS CPSs with B2 B subsets (including both marginal zone and follicular B cells). However, both B1 and B2 subsets were affected by the immunosuppressive effect of *N. meningitidis* group C CPS [64], and in vivo tolerance induction by a sialylated polymer targeted, at least in part, follicular B cells [62]. The dose of the CPS might also influence its immunosuppressive effect, with higher doses being more suppressive, as previously reported [64]. Yet, in our hands, an increase in the CPS dose failed to reveal a modulatory effect in vitro (unpublished observations). Finally, we have no explanation for the apparent tendency of

our CPS preparations to increase in vitro B cell responsiveness to BAFF, an effect that seems to be favored by the presence of sialic acid. This question warrants further investigations.

Overall, we have found that sialylated CPSs expressed from two distinct serotypes of two different Gram-positive streptococci, *S. suis* and GBS, are poorly-immunogenic antigens. The inability of host to mount an effective Ab response specifically directed against purified CPSs of these two pathogens do not seem to be explained by the capacity of sialic acid on its own to have instructed immune cells to recognize CPS as a "self-antigen" or to actively dampen B cell functions. This is further evidence by the lack of suppressive effect on a TD response in vivo (for instance, the co-injection of purified *S. suis* or GBS CPSs with OVA did not significantly dampen the generation of OVA-specific Abs in mice). Our results also suggest that terminal sialic acid of Gram-positive bacterial CPSs may not exert a marked inhibitory effect on the anti-CPS humoral response contrary to sialylated Gram-negative bacterial CPSs. Indeed, in the case of GBS type III, this sugar is part of the immunodominant epitope. The failure of intact bacteria, where CPS is associated with a particulate structure with numerous adjuvanting moieties, to generate an optimal anti-CPS response, including specific IgG production, might be due to intrinsic biochemical properties of these CPSs that could be related or not to the presentation of CPS on the bacterial surface. Their identification will be a valuable tool for a better understanding of the immunopathogenesis of *S. suis* and GBS infections, as well as for the development of efficient strategies to fight against these bacteria.

Materials and Methods

Mice

Female 5- to 8-week-old C57BL/6 mice (Charles River Laboratories) were acclimatized to standard laboratory conditions with free access to water and rodent chow. All experiments were conducted in accordance with the guidelines and policies of the Canadian Council on Animals 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 (Protocol # 2016-Rech-1399 and 1523) [72].

Bacterial Strains and Growth Conditions

Encapsulated virulent *S. suis* serotype 2 strain P1/7 isolated from a pig with meningitis [73], *S. suis* serotype 14 strain DAN13730 isolated from a human with meningitis [74], GBS serotype III strain COH-1 isolated from an infant with sepsis and meningitis [75] and GBS serotype V strain CJB111 (ATCC BAA-23) isolated from a neonate with septicemia were used for experimental infections. These strains were already used in earlier studies and were cultured as previously described [5,10,40]. Briefly, bacteria were grown overnight onto sheep blood agar plates at 37 °C and isolated colonies were cultured in 5 mL of Todd–Hewitt broth (THB; Becton Dickinson, Mississauga, ON, Canada) for 8 h at 37 °C. Then, 10 µL of a 10^{-3} dilution of 8 h-cultures were transferred into 30 mL of THB and incubated for 16 h (*S. suis*) or 12 h (GBS) at 37 °C. Stationary phase bacteria were washed in phosphate-buffered saline (PBS, pH 7.3). The bacterial pellet was then resuspended in THB and adjusted to the desired concentrations. Non-encapsulated mutant strains *ΔcpsF* derived from strain P1/7, Δ*cps14b* derived from strain DAN13730 [5], Δ*cpsE* derived from strain COH-1 [9] and Δ*cpsE* derived from strain CJB111 [10] were used as coating for enzyme-linked immunosorbent assay (ELISA) of non-capsular, mainly protein-specific Ab response as described previously [41]. For both encapsulated and non-encapsulated strains, aliquots of final bacterial suspensions were plated using an Autoplate 4000 automated spiral plater (Spiral Biotech, Norwood, MA, USA) onto sheep blood agar plates (Oxoid, Nepean, ON, Canada), and colonies were accurately counted after overnight incubation at 37 °C.

CPS Purification and Desialylation

The native CPSs of *S. suis* type 2, *S. suis* type 14, GBS type III and GBS type V were purified as previously described [39]. Desialylated CPSs were obtained by mild acid hydrolysis [39]. Each purified CPS was subjected to rigorous physicochemical and immunologic quality control tests to ensure the identity and the purity of the CPS, the preservation of epitope recognition and the absence of sialic acid in the desialylated preparations [39]. Briefly, the absence of nucleic acid and protein contamination was confirmed by spectrophotometry; each CPS was analyzed by nuclear magnetic resonance (NMR), and the monosaccharide composition was confirmed by methanolysis followed by

acetylation and analysis by gas chromatography, either with flame ionization detection or coupled to mass spectrometry. The presence or absence of sialic acid was verified by NMR and by an enzyme-linked lectin assay. Finally, the preservation of epitope recognition was confirmed by Dot-ELISA using CPS-specific sera, as previously described [39]. Similar yields of CPS production were obtained with the four strains per liter of culture under normalized O.D. $_{600nm}$ values of 0.8. The average CPS yield was of 6.8 ± 2.6 mg/L for *S. suis* type 2, 6.4 ± 1.9 mg/L for *S. suis* type 14, 6.3 ± 4.2 mg/L for GBS type III, and 6.4 ± 1.5 mg/L for GBS type V.

Bacterial Infections

A live suspension of 2×10^7 CFU of strain P1/7, 5×10^6 CFU of strain DAN13730, 2×10^6 CFU of strain COH-1 or 10^4 CFU of strain CJB111 was administrated intraperitoneally (i.p.) to mice on Day 0. Optimal bacterial doses were determined in standardization pre-trials and vary depending on the pathogenicity of each strain (data not shown). Negative control mice were injected with vehicle solution (sterile THB). Blood bacterial loads were assessed by collecting a 5-µL blood sample from the tail of each mouse at 12 h post-infection. Proper dilutions were plated and bacterial numbers counted as described above. Samples were taken on Days 7, 14 and 21 post-infection. To reduce the number of mice, on Days 7 and 14, blood samples were collected from the tail vein of each mouse for anti-CPS Ig titration. On Day 21, mice were euthanized and bled for anti-CPS and anti-protein Ig titration. The numbers of animals included in all experiments are detailed in the figure legends.

Immunizations

In a first set of experiments aimed to compare the immunogenicity of purified *S. suis* types 2, 14 and GBS types III and V CPSs, as well as to evaluate the influence of the presence of sialic acid, mice were immunized subcutaneously (s.c.) twice at 3-week intervals with 2 µg of each native or desialylated CPS emulsified with STIMUNE® (Prionics, La Vista, NE, USA) following the manufacturer's recommendations, in a final volume of 100 µL. STIMUNE® is a water-in-oil adjuvant composed of purified and defined mineral oil (Markol 52) with Span 85 and Tween 85 as emulsifiers, which has been used as a good alternative to Freund's adjuvant

for weak immunogens in animals [76]. The dose of CPS was determined according to the literature [34,46,55,77], as well as after dose-response experiments conducted with the purified *S. suis* type 2 CPS [54]. The placebo group received STIMUNE® alone. On Days 7, 14, 21, 28, 35 and 42 post-immunization, blood samples were collected from the tail vein of each mouse for anti-CPS Ig titration.

In selected experiments aimed to evaluate the adjuvant effect of exogenous TLR agonists on the CPS-specific Ab response, mice were immunized s.c. with 2 µg of purified native or desialylated *S. suis* type 2 CPS in 100 µL of PBS on Day 0, followed by an administration of 80 µg of CpG 1826 ODNs (InvivoGen, San Diego, CA, USA) in 100 µL of PBS via the same route two days after. A group of mice similarly immunized with 2 µg of PS3 (ATCC 31-X), followed by 80 µg of CpG 1826 ODNs two days after, was included for comparative purpose. This immunization protocol was chosen based on the literature [34,46]. The control group received native or desialylated *S. suis* type 2 CPS or PS3 on Day 0 followed by PBS injection on Day 2. Two placebo groups receiving only PBS or CpG 1826 ODNs were also included. On Days 0 (before immunization), 7, 14 and 21 post-immunization, blood samples were collected from the tail vein of each mouse for anti-CPS Ig titration. On Day 5 [34,46], randomly selected mice of each group were euthanized, and spleens were removed to enumerate CPS-specific ASCs by enzyme-linked ImmunoSpot (ELISpot) (see below).

A last set of experiments aimed to evaluate the immunomodulatory effect of purified *S. suis* types 2, 14 and GBS types III and V CPSs on the development of the OVA-specific Ab response. On Day 0, mice were immunized i.p. with 10 µg of OVA (Sigma-Aldrich, Oakville, ON, Canada) in association with 2 µg of each individual native CPS in a final volume of 100 µL of PBS. A second dose with the same preparation was given on Day 21 post-primary immunization. Control group received OVA alone on Days 0 and 21. A placebo group receiving PBS alone was also included. On Days 7, 14, 21 and 35 post-immunization, blood samples were collected from the tail vein of each mouse for anti-OVA Ig titration. The numbers of animals included in the experiments are detailed in the figure legends.

In Vitro B Cell Stimulation Assay

Untouched B cells were purified from the spleen of naive mice by negative selection using the B Cell Isolation Kit microbeads and magnetically activated cell sorting (MACS; Miltenyi Biotech, Auburn, CA, USA) according to the manufacturer's instructions and resuspended at 10^6 cells/mL in complete medium, consisting of RPMI 1640 supplemented with 10% 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 (Gibco, Invitrogen, Burlington, ON, Canada). The enriched B cells had >95% purity as determined by fluorescence-activated cell sorter (FACS) analysis using anti-CD19 staining (data not shown).

To evaluate the immunomodulatory effect of purified *S. suis* types 2, 14 and GBS types III and V CPSs on the ability of BAFF or CpG 1826 ODNs to stimulate B cells, these cells were co-incubated with 20 μg/mL of each individual native or desialylated CPS and 1 μg/mL of BAFF along with 50 ng/mL of IL-4 (BioLegend, San Diego, CA, USA), as reported [64]. Similarly, in a second set of experiments, B cells were co-incubated with 1 μg/mL of CpG 1826 ODNs and 20 μg/mL of each individual native or desialylated CPS. After 7 days, supernatants were collected for quantification of the Ig secretion. In some experiments, B cells were pre-stimulated with CPS for 24 h prior to incubation with BAFF/IL-4 or CpG 1826 ODNs for 6 days. Cells stimulated with BAFF/IL-4 alone or CpG 1826 ODNs alone (without CPS) served as positive controls. In both set of experiments, the Ig secretion in the supernatants of B cells incubated with medium alone or each purified CPS alone was also measured as an indication of the basal Ig secretion by B cells in the absence of CpG 1826 ODNs or BAFF/IL-4 stimulation.

ELISA

Samples from In Vivo Assays

For titration of protein-specific Abs in *S. suis*- or GBS-infected mice, Polysorb immunoplates (Canadawide Scientific, Toronto, ON, Canada) were coated with the respective non-encapsulated mutant strains as described previously [41]. Albeit a response to other cell wall components might be detected, proteins are the dominant antigens present at the surface

of non-encapsulated mutants, and thus, the detected response in the ELISA is labelled as "anti-protein" throughout the study for simplicity. After washes in PBS containing 0.05% Tween 20 (PBS-T), mouse sera were serially diluted (two-fold) in PBS-T (starting with a dilution of 1/50) and incubated 1 h at room temperature (RT). After washes in PBS-T, plates were incubated with peroxidase-conjugated goat anti-mouse total Ig (IgG plus IgM), IgG (Jackson Immunoresearch, West Grove, PA, USA) or IgM (Southern Biotech, Birmingham, AL, USA) Abs for 1 h at RT. Plates were developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Invitrogen) substrate, and the enzyme reaction was stopped by the addition of 0.5 M H_2SO_4. Absorbance was read at 450 nm with an ELISA plate reader. The reciprocal of the last serum dilution that resulted in an optical density ($OD_{450\ nm}$) equal or lower of 0.2 (cut-off) was considered the titer of that serum. When the $OD_{450\ nm}$ of the first dilution of a serum was lower than the cut-off, its titer was arbitrary fixed to 50. For titration of CPS- or OVA-specific Abs, a solution of 2 µg/mL of purified native *S. suis* type 2 or 14 or GBS type III or V CPS in carbonate buffer 0.1 M pH 9.6, or PS3 or OVA in PBS (100 µL/well) was added overnight on Polysorb immunoplates at 4 °C. Plates were then blocked 1 h at RT with 1% BSA in PBS before the addition of mouse serum dilutions. CPS- and OVA-specific Ab titers were determined as described above.

To control inter-plate variations, we added an internal reference positive control to each plate. For *S. suis* or GBS CPS- or protein-specific ELISA, this control was a pool of sera from mice hyperimmunized i.p. with 10^9 CFU of heat-killed whole *S. suis* or GBS of the respective serotypes. For PS3-specific ELISA, this control was a pool of sera from mice immunized s.c. with 0.5 µg of PS3 followed by 80 µg of CpG 1826 ODNs two days after. For OVA-specific ELISA, this control was a pool of sera from mice co-immunized i.p. with 10 µg of OVA plus 20 µg of CpG 1826 ODNs. Reaction in TMB was stopped when an $OD_{450\ nm}$ of 1 was obtained for the positive control. Optimal dilutions of the coating antigen, the positive internal control sera and the peroxidase conjugated anti-mouse Abs were determined during preliminary standardizations.

Samples from In Vitro Assays

The total IgM and IgG levels in B cell culture supernatants were measured with a Mouse IgM or a Mouse IgG ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA), respectively, according to the manufacturer's instructions. Two-fold dilutions of mouse reference IgM or IgG serum (Bethyl Laboratories) 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 Ig.

ELISpot

Spleens from mice immunized with native or desialylated *S. suis* type 2 or PS3 (with or without CpG 1826 ODNs) or from placebo groups (used as negative controls) were harvested 5 days post-immunization and pressed gently through a sterile fine wire mesh, as described above. After incubation with NH_4Cl lysing buffer (eBioscience, San Diego, CA, USA) to remove red blood cells, total splenocytes were resuspended in complete medium. Assays were performed using 96-well MultiScreen high protein binding immunobilon-P membrane plates (Millipore, Billerica, MA, USA) coated with 2 μg/mL of purified native *S. suis* type 2 or PS3 in PBS, overnight at 4 °C. Plates were then washed in PBS and blocked for 2 h at 37 °C with complete medium. Splenocytes were serially diluted (two-fold) in complete medium (starting with a concentration of 5×10^6 cells/mL) and 100 μL/well of each dilution was incubated on the plates for 24 h at 37 °C. Subsequently, plates were washed in PBS-T and incubated with peroxidase-conjugated goat anti-mouse total Ig (IgG plus IgM) Ab for 2 h at RT. Plates were developed with TMB substrate. Spots were counted using a CTL ImmunoSpot S4 ultraviolet Analyzer (Cellular Technology Limited, Cleveland, OH, USA). The background was subtracted, and data were expressed as the number of ASCs/10^6 total splenocytes. Sample were considered positive if the number of ASCs was >10/10^6 total splenocytes and 2 SD above the negative control. Optimal dilutions of the coating antigen and the peroxidase conjugate were determined during preliminary standardizations.

Statistical Analysis

Normality of data were evaluated using the Shapiro–Wilk test. Accordingly, the unpaired *t*-test (or the non-parametric Mann–Whitney rank sum test) was used in in vitro and in vivo studies. Analysis of variance (ANOVA) was used to analyze the significance of total anti-CPS Ab titers in Figures 1 and 2, as well as the significance of Ab titers in Figures S2, S3, S6 and A1. Data were analyzed with the Sigma Plot System (v11.0; Systat Software). A $p < 0.05$ was considered as statistically significant.

Supplementary Materials: The following are available online at www.mdpi.com/link: Figure S1: Blood bacteremia of surviving mice after infection with *S. suis* serotype 2, *S. suis* serotype 14, GBS serotype III or GBS serotype V. Figure S2: Titration of CPS-specific antibodies in mice after immunization with purified native GBS serotype III. Figure S3: Titration of CPS-specific antibodies in mice after primary and secondary immunization with purified native or desialylated *S. suis* serotype 2, *S. suis* serotype 14, GBS serotype III or GBS serotype V CPS. Figure S4: Adjuvant effect of CpG ODNs on the CPS-specific antibody response in mice immunized with purified *S. pneumoniae* serotype 3 CPS (PS3). Figure S5: In vitro immunomodulatory effect of purified native or desialylated *S. suis* serotype 2, *S. suis* serotype 14, GBS serotype III or GBS serotype V CPS on the Ig secretion by naive B cells induced by CpG ODNs. Figure S6: Titration of CPS-specific antibodies in mice after immunization with purified native or desialylated *S. suis* serotype 2 CPS.

Acknowledgments: This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a grant to Mariela Segura (#342150). We thank Fondation Roland-Beaulieu and Fondation Centre de cancérologie Charles-Bruneau for key infrastructure support. Insaf Salem Fourati was the recipient of a doctoral scholarship from Fonds de la recherche du Québec-santé (FRQS).

Appendix A

In order to study the specific role of sialic acid linkage in the immunogenicity of GBS type III CPS, we substitute GBS type III α2,3-sialyltransferase *cpsK* gene (Accession Number

AAD53072) by *S. suis* α2,6-sialyltransferase gene *cpsN* (Accession Number CAR45180), as recently described [78]. CPS expression in the α2,6 exchanged-GBS mutant was confirmed by a cell surface hydrophobicity test and transmission electron microscopy, as reported [10]. The CPS was purified as previously described [39] and subjected to rigorous physicochemical and quality control tests to ensure the identity and the purity of the CPS, as well as the presence of α2,6 sialic acid linkage instead of α2,3 linkage by nuclear magnetic resonance spectroscopy [78]. Purified CPS was used to immunize mice as described in the Materials and Methods (Section 4.5).

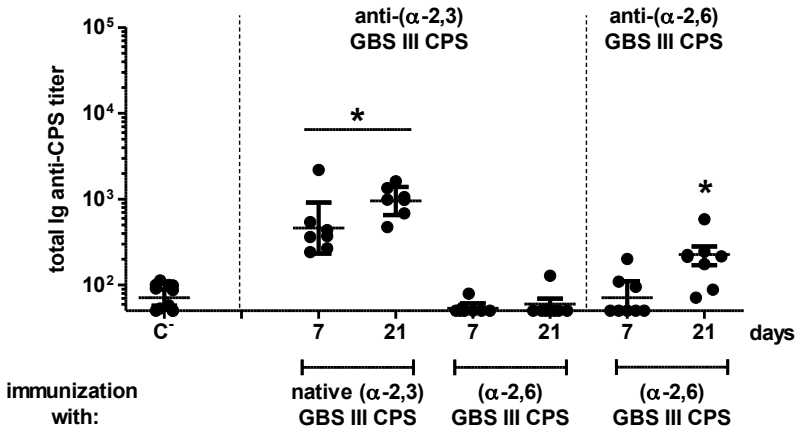

Figure A1. Titration of CPS-specific antibodies in mice after primary immunization with (α-2,3) or (α-2,6) GBS serotype III CPS. Mice (n = 8) were immunized with 2 μg of (α-2,3) or (α-2,6) GBS serotype III CPS emulsified with STIMUNE® on Day 0. Total Ig (IgG plus IgM) anti-(α-2,3) (left) or anti-(α-2,6) (right) GBS serotype III CPS titers were determined by ELISA on Days 7 and 21. "C^-" represents a pool of control mice (n = 3) injected with STIMUNE® only, whose anti-(α-2,3) or anti-(α-2,6) GBS serotype III CPS titers were evaluated on Days 7 and 21. Data from individual mice are presented, including the geometric mean with 95% confidence interval. * Statistically significant difference ($p < 0.05$) in comparison to the C^- group.

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ANNEXE; ARTICLE X

Type I Interferon induced by *Streptococcus suis* serotype 2 is strain-dependent and may be beneficial for host survival

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Soumis dans: Frontiers in Immunology (Avril **2017**).

Détails sur le rôle du candidat dans la conception de l'article :
Je suis coauteur de l'article. J'ai préparé les échantillons pour les expériences de microscopie électronique à transmission et j'ai également analysé et préparé les images des photographies électroniques inclues dans cette article.

Abstract

Streptococcus suis serotype 2 is an important porcine bacterial pathogen and emerging zoonotic agent mainly responsible for sudden death, septic shock, and meningitis, with exacerbated inflammation being a hallmark of the infection. However, serotype 2 strains are genotypically and phenotypically heterogeneous, being composed of a multitude of sequence types (ST) whose virulence greatly varies: the virulent ST1 (Eurasia), highly virulent ST7 (responsible for the human outbreaks in China), and intermediate virulent ST25 (North America) are the most important worldwide. Even though type I interferons (IFNs) are traditionally associated with important anti-viral functions, recent studies have demonstrated that they may also play an important role during infections with extracellular bacteria. Up-regulation of IFN-β levels was previously observed in mice following infection with this pathogen. Consequently, the implication of IFN-β in the *S. suis* serotype 2 pathogenesis, which has always been considered a strict extracellular bacterium, was evaluated using strains of varying virulence. This study demonstrates that intermediate virulence strains are significantly more susceptible to phagocytosis than virulent strains. Hence, subsequent localization of these strains within the phagosome results in recognition of bacterial nucleic acids by Toll-like receptors 7 and 9, leading to activation of the interferon regulatory factors 1, 3, and 7 and production of IFN-β. Type I IFN, whose implication depends on the virulence level of the *S. suis* strain, is involved in host defense by participating in the modulation of systemic inflammation, which is responsible for the clearance of blood bacterial burden. As such, when induced by intermediate, and to a lesser extent, virulent *S. suis* strains, type I IFN plays a beneficial role in host survival. The highly virulent ST7 strain, however, hastily induces a septic shock that cannot be controlled by type I IFN, leading to rapid death of the host. A better understanding of the underlying mechanisms involved in the control of inflammation and subsequent bacterial burden could help to develop control measures for this important porcine and zoonotic agent.

Introduction

Streptococcus suis is an important porcine bacterial pathogen and emerging zoonotic agent mainly responsible for sudden death (pigs), septic shock (humans), and meningitis (both species) (1). Of the different described serotypes based on the presence of the capsular polysaccharide (CPS) or its respective genes, serotype 2 is regarded as not only the most widespread worldwide, but also the most virulent, responsible for the majority of porcine and human cases of infection (2). However, serotype 2 strains are genotypically and phenotypically heterogeneous, being composed of a multitude of sequence types (STs), as determined by multilocus sequence typing, whose distribution greatly varies worldwide (2). Virulence of the most important STs (ST1, ST7, and ST25) has been evaluated using mouse models of infection (3, 4). Indeed, the ST7 strain responsible for the human outbreaks of 1998 and 2005 in China is highly virulent whereas European ST1 strains are virulent; on the other hand, ST25 strains, typically recovered in North America, are of intermediate virulence (3, 5).

Of the various virulence factors described for *S. suis*, the CPS, suilysin (SLY), and cell wall modifications have been demonstrated to play important roles (6, 7). Indeed, the CPS, which is antigenically identical for all serotype 2 strains, is a critical factor implicated in a multitude of functions, most importantly in resistance to phagocytosis by innate immune cells (8-12); its presence also masks bacterial surface proteins responsible for host cell activation (12, 13). Meanwhile, the SLY, a cholesterol-dependent cytolysin similar to the pneumolysin of *Streptococcus pneumoniae*, is responsible for causing cell toxicity and inducing pro-inflammatory cytokines (12, 14). This toxin is present in serotype 2 ST1 and ST7 strains, but not in ST25 strains (14, 15). Cell wall modifications, such as the D-alanylation of the lipoteichoic acid and N-deacetylation of the peptidoglycan, of a ST1 strain were shown to interfere with host defense and to be partially responsible for cell activation (12, 16, 17). Finally, several cell-wall associated proteins, mainly reported for ST1 and ST7 strains, have also been described as critical virulence factors, though many of these remain controversial in the literature (7).

Recognition of *S. suis* by innate immune cells involves a multitude of membrane-associated and cytoplasmic receptors (6, 18). Of these, the Toll-like receptor (TLR) pathway is

implicated in recognition of *S. suis* by phagocytic cells, including dendritic cells (DCs) and macrophages (19). Abrogation of MyD88, the adaptor protein central to the TLR pathway, results in near complete lack of pro-inflammatory cytokine production *in vitro* following infection with *S. suis* (13, 20). Furthermore, being a classical extracellular pathogen, recognition of *S. suis* has been mostly associated with surface TLRs, where TLR2, in cooperation with TLR6, plays a predominant role (13, 20, 21). Pathogen recognition by TLRs classically results in the production of pro-inflammatory cytokines via the NF-κB or interferon (IFN) pathways (19). Pathways involved in activation of NF-κB by *S. suis* have been somewhat described in recent years (20, 22), while those regarding the IFN pathways are less known, having mainly focused on type II IFN (3, 23). Nonetheless, it was recently demonstrated that expression levels of the type I IFN, IFN-β, but not those of IFN-α, are up-regulated *in vivo* following infection with *S. suis* serotype 2 (3). Moreover, this up-regulation of IFN-β was significantly higher in mice infected with an intermediate virulent ST25 strain than in those infected with either a virulent ST1 strain or the highly virulent ST7 strain responsible for the human outbreaks (3). However, no other study has addressed the production of type I IFN or its role during the *S. suis* serotype 2 infection.

Type I IFN regroups various members of the IFN family of which IFN-α, composed of sixteen different subtypes, and IFN-β, its most potent member, are the best characterized (24). Classical production of these cytokines is the result of endosomal TLR (TLR3, TLR7, and TLR9 in mice) activation, which leads to phosphorylation and translocation of interferon regulatory factors (IRFs) to the nucleus (25). Though IRF1 and IRF3/IRF7 are usually associated with type II and type I IFN, respectively, all three can result in transcription of type I IFNs (24, 25). Following their production, both IFN-α and IFN-β bind a common heterodimeric receptor, the IFN-αβ receptor (IFNAR) (25). Binding to this receptor activates the JAK/STAT pathway, transcription of various genes associated with host defense, and modulation of the inflammatory response (25).

Even though type I IFNs are traditionally associated with important anti-viral functions, recent studies have demonstrated that they may also play an important role, particularly for IFN-β, during bacterial infections, including pathogenic streptococci (25, 26). However, their role,

whether beneficial or detrimental, may depend on the bacterial species and/or infection model (27-31). As aforementioned, little information is available regarding type I IFN, and more specifically IFN-β, during the *S. suis* infection, which is considered a strict extracellular pathogen. Consequently, its implication in the *S. suis* serotype 2 pathogenesis was evaluated using strains of varying virulence. Herein, we demonstrated that following phagocytosis by DCs, to which intermediate virulence strains are more susceptible, *S. suis* is located within the phagosome where bacterial nucleic acids are recognized by TLR7 and TLR9, leading to activation of IRF1, IRF3, and IRF7 and production of IFN-β. When induced by intermediate, and to a lesser extent, virulent *S. suis* strains, but not by a highly virulent strain, type I IFN plays a beneficial role, being involved in the control of blood bacterial burden via modulation of systemic inflammation.

Results

Strain- and cell type-dependent induction of IFN-β by *S. suis* serotype 2. DCs and macrophages have not only been demonstrated to be important for IFN-β production during bacterial infections, but were also shown to produce high levels of other pro-inflammatory cytokines following infection with *S. suis* (12, 13, 20, 41). Consequently, the capacity of these cells to produce IFN-β following infection with three different *S. suis* serotype 2 strains (highly virulent ST7 strain SC84, virulent ST1 strain P1/7, and intermediate virulent ST25 strain 89-1591) was evaluated. This cytokine was chosen as a representative of type I IFN since *S. suis* was previously demonstrated to up-regulate levels of IFN-β expression *in vivo*, but not those of IFN-α (3). Moreover, DCs and macrophages have been demonstrated to mainly produce IFN-β, but only low levels of IFN-α, following infection with pathogenic streptococci (27, 29, 37). As shown in **Fig. 1A**, DCs expressed low levels of IFN-β 3 h after infection with *S. suis* (**Fig. 1A**). However, expression levels quickly and significantly increased, peaking at 6 h ($p < 0.05$). Importantly, levels induced by the intermediate virulent ST25 strain 89-1591 were significantly higher ($p < 0.001$) than those induced by the two more virulent strains. These levels were not specific to 89-1591, since similar high levels were also obtained using two other intermediate virulent ST25 strains (**Figure S1 in Supplementary Material**). In contrast, IFN-β expression levels of *S. suis*-infected macrophages remained relatively low and unchanged, regardless of the incubation time and strain used (**Fig. 1B**).

Indeed, IFN-β expression levels by macrophages were significantly lower than those by DCs and this, for all three strains tested ($p < 0.01$). A clear induction of IFN-β expression was detected with the positive control, poly(I:C), indicating that the low response observed with *S. suis* was a consequence of the stimulus rather than the cells (**Fig. 1B**).

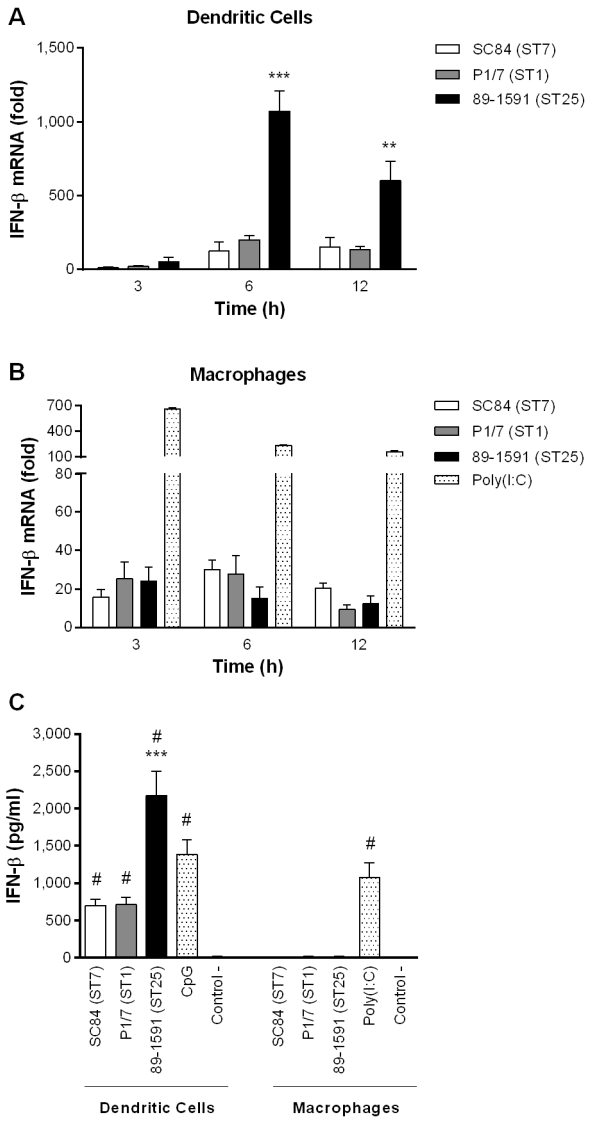
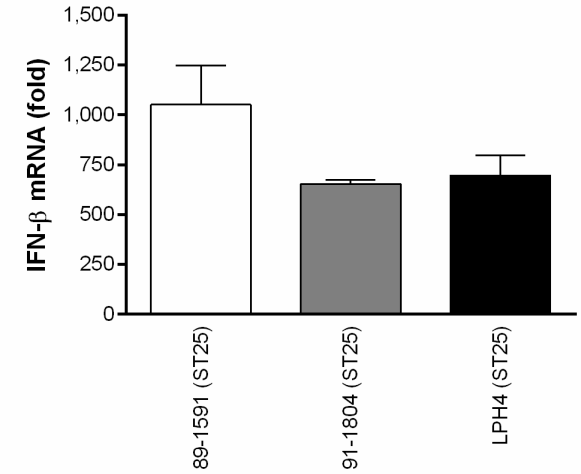


Figure 1. Dendritic cells (DCs) produce higher levels of IFN-β than macrophages following infection with *S. suis* serotype 2. IFN-β mRNA expression kinetics, measured by RT-qPCR, following infection of DCs (A) and macrophages (B), with the highly virulent ST7 strain SC84, virulent ST1 strain P1/7, and intermediate virulent ST25 strain 89-1591. IFN-β protein production by DCs and macrophages was measured by ELISA 24 h following infection with the different *S. suis* strains (C). Data represent the mean ± SEM from four independent experiments. ** ($p < 0.01$) and *** ($p < 0.001$) indicate a significant difference between 89-1591 and P1/7 or SC84; # ($p < 0.001$) between

S. suis or CpG and the negative control (control -) for DCs or between poly(I:C) and negative control (control -) for macrophages.



Supplementary Figure S1. Intermediate virulent *S. suis* ST25 strains induce high levels of IFN-β expression by dendritic cells. IFN-β mRNA expression by dendritic cells 6 h following infection with the intermediate virulent ST25 strains 89-1591, 91-1804, and LPH4. Virulence of strains 91-1804 and LPH4 was previously described.

In order to evaluate if these differences in expression between cell types were also observed at the protein level, IFN-β was measured in the supernatant of cells 24 h after infection by ELISA (**Fig. 1C**). Indeed, IFN-β mRNA expression and protein production correlated. Results demonstrated that only DCs produce important protein levels of IFN-β following infection with *S. suis* serotype 2, which were significantly higher than control cells ($p < 0.001$) (**Fig. 1C**). However, as with mRNA expression, the intermediate virulent strain 89-1591 induced significantly higher protein levels of IFN-β by DCs than the other two *S. suis* strains ($p < 0.001$). Meanwhile, macrophages produced significant levels of IFN-β when stimulated with poly(I:C) ($p < 0.001$), but not following *S. suis* infection, confirming results observed at the transcriptional (mRNA) level (**Fig. 1C**). Based on these results, all subsequent experiments in this study were performed using DCs.

The presence of capsular polysaccharide interferes with *S. suis*-induced IFN-β expression by dendritic cells, while the suilysin (when present) is partially responsible for activation. Of the different described virulence factors for *S. suis* serotype 2, the presence of the CPS,

SLY, and cell wall modifications have been reported to modulate and/or participate in cytokine production by DCs (12). Consequently, their role in the induction of IFN-β expression by DCs was evaluated using isogenic mutants (**Fig. 2**). Absence of the CPS in a ST1 strain resulted in a significant increase of IFN-β expression by DCs ($p < 0.001$), suggesting that its presence interferes with cell activation. On the other hand, absence of the SLY, which is a pore-forming toxin present in ST1 and ST7 strains, resulted in a significant decrease of IFN-β expression ($p < 0.01$). Meanwhile, cell wall modifications (D-alanylation of the lipoteichoic acid and the N-acetylation of the peptidoglycan) did not modulate *S. suis*-induced IFN-β by DCs. Interestingly, levels of IFN-β induced by the ST1 strain 31533 were very similar to those observed with the prototype strain P1/7.

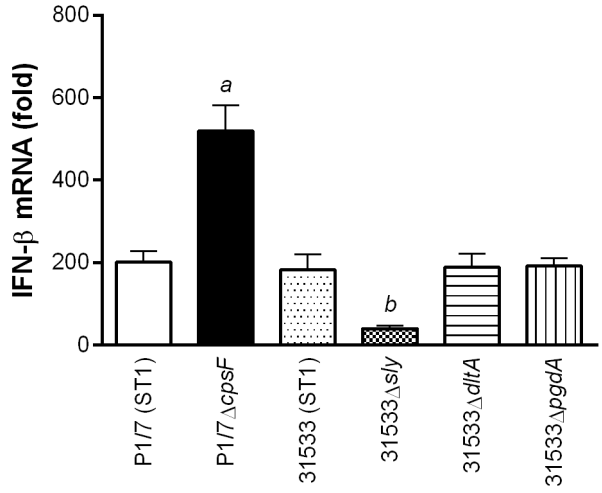


Figure 2. The presence of the capsular polysaccharide interferes with *S. suis*-induced IFN-β expression by dendritic cells, while the suilysin is partially responsible for its activation. Role of the capsular polysaccharide (CPS), suilysin (SLY), and cell wall modifications (D-alanylation of the lipoteichoic acid, Δ*dlta* or N-deacetylation of the peptidoglycan, Δ*pgdA*) in IFN-β mRNA expression by dendritic cells 6 h following infection with the wild-type or mutant *S. suis* strains. Data represent the mean ± SEM from four independent experiments. *a* ($p < 0.001$) indicates a significant difference between P1/7 and P1/7Δ*cpsF*; *b* ($p < 0.01$) between 31533 and 31533Δ*sly*.

Recognition of *S. suis* by the Toll-like receptor pathway is required for IFN-β induction in dendritic cells. The TLR pathway has been traditionally associated with IFN-β production following pathogen recognition by the endosomal TLRs (TLR3, TLR7, and TLR9 in mice) (25). However, being considered a classical extracellular pathogen, recognition of *S. suis* has

been mostly associated with surface TLRs (TLR1, TLR2, and TLR6) (13, 20). Consequently, the role of the TLR pathway in *S. suis*-induced IFN-β by DCs was evaluated. In the absence of the adaptor protein MyD88, used by the majority of TLRs, a significant decrease of IFN-β expression by DCs ($p < 0.001$) was observed with the three *S. suis* strains, corresponding to a near complete abrogation (**Fig. 3A**). This result suggests that *S. suis*-induced IFN-β expression by DCs is overwhelmingly MyD88-dependent since only 5 to 10% of expression remained independent of MyD88 (**Fig. 3A**).

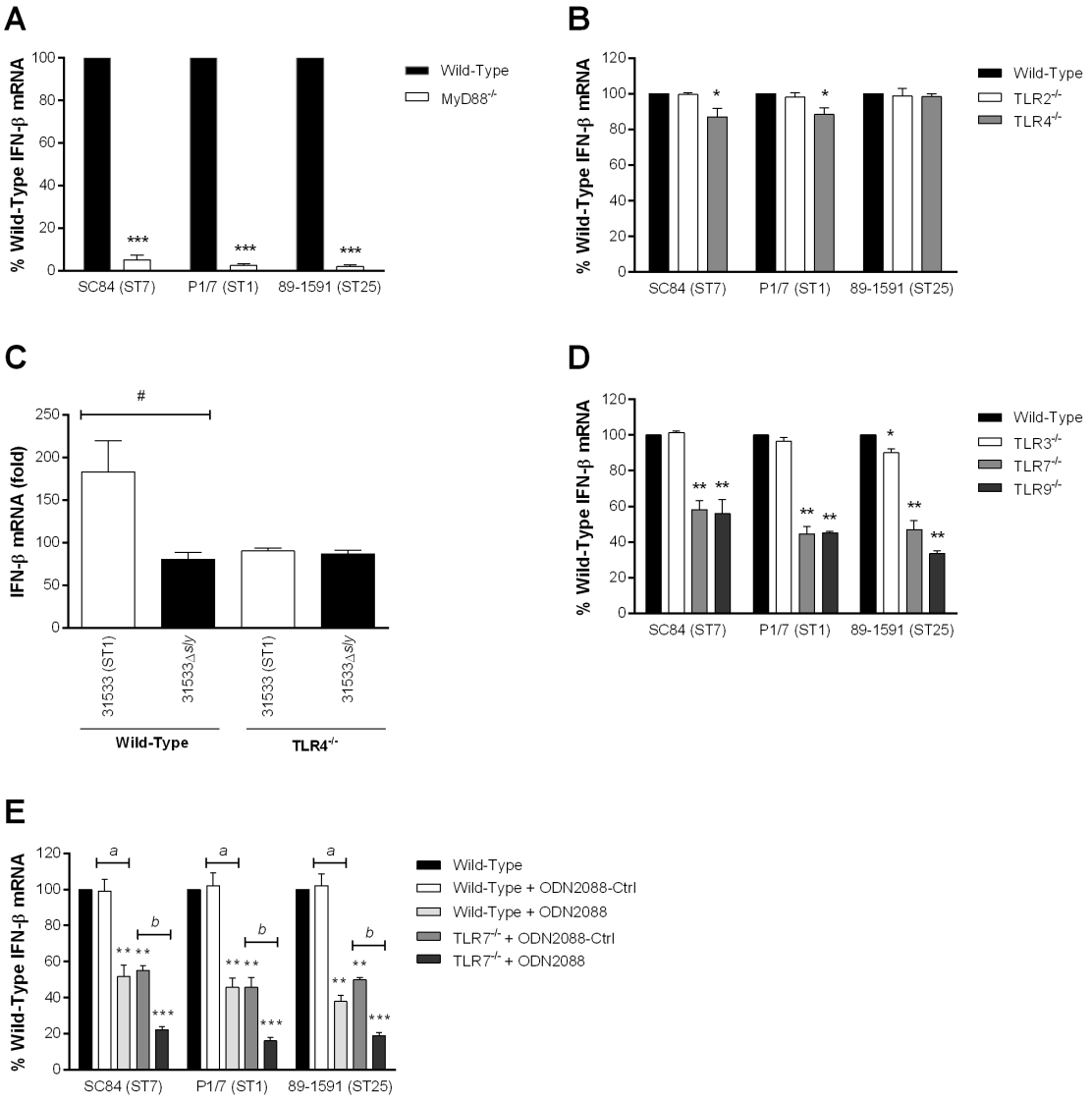
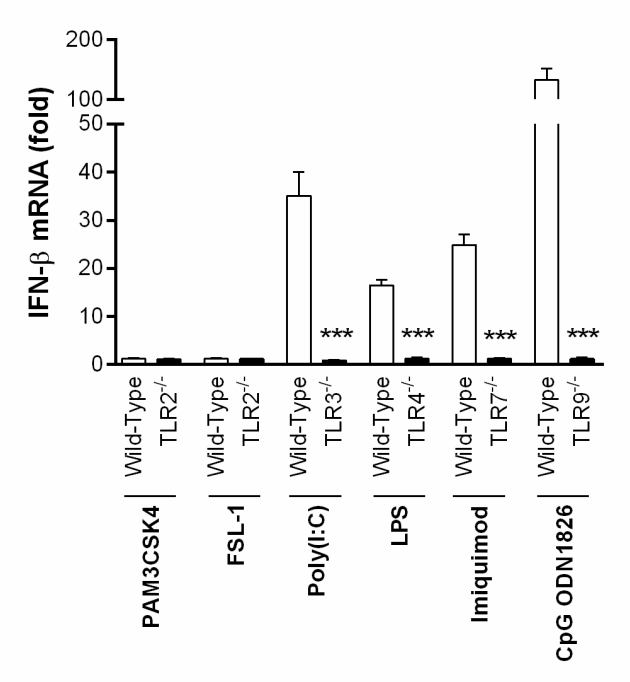


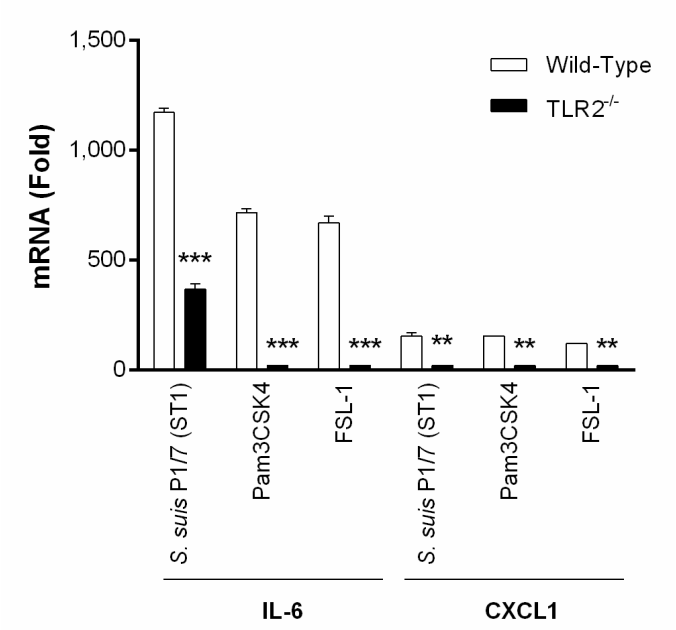
Figure 3. Recognition of *S. suis* by the Toll-like receptor (TLR) pathway is required for induction of IFN-β expression by dendritic cells (DCs). IFN-β mRNA expression induced by the different *S. suis* strains 6 h following infection of DCs deficient for MyD88 (A), TLR2 or TLR4 (B), or for the endosomal TLR3, TLR7, or TLR9 (D). The suilysin (SLY) is responsible for TLR4-dependent IFN-β expression by DCs (C). The cooperative role of TLR7 and TLR9 was evaluated using wild-type or TLR7$^{-/-}$ cells pretreated with the TLR9 antagonist ODN2088 or its control, ODN2088-Ctrl, resulting in TLR7$^{-/-}$ cells non-responsive for TLR9 (dual deficiency) (E). Data represent the mean ± SEM from three or four independent experiments. * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$) indicates a significant difference between expression by wild-type and deficient DCs; # ($p < 0.01$) between 31533 and 31533Δ*sly*; *a* ($p < 0.01$) between expression by wild-type DCs pretreated with ODN2088-Ctrl or ODN2088; *b* ($p < 0.01$) between expression by TLR7$^{-/-}$ DCs pretreated with ODN2088-Ctrl or ODN2088.

Given the near complete MyD88-dependence of *S. suis*-induced IFN-β by DCs and the fact that the surface TLR2 (13, 20) and, possibly TLR4 via the SLY (42), may be implicated in recognition of this pathogen, their role in *S. suis*-induced IFN-β by DCs was evaluated. TLR2 was not implicated in IFN-β expression by DCs following *S. suis* infection, regardless of the strain used (**Fig. 3B**). Moreover, it was not possible to induce IFN-β expression following stimulation of wild-type DCs with PAM3CSK4 (for TLR1/2) and FSL-1 (for TLR2/6) (43), which are synthetic bacterial TLR2 ligands frequently used for most cell types (**Figure S2 in Supplementary Material**).



Supplementary Figure S2. Ligands of the different Toll-like receptors (TLRs) evaluated in this study, with the exception of TLR2, induce IFN-β by dendritic cells. IFN-β mRNA expression by wild-type and deficient dendritic cells 6 h following activation with the different TLR ligands: 1 µg/mL PAM3CSK4 (TLR1/2), 1 µg/mL FSL-1 (TLR2/6), 10 µg/mL poly(I:C) (TLR3), 100 ng/mL lipopolysaccharide (LPS) (TLR4), 5 µg/mL imiquimod (TLR7), and 1 µM CpG ODN1826 (TLR9). Data represent the mean ± SEM from three or four independent experiments. *** ($p < 0.001$) indicates a significant difference in IFN-β expression by wild-type and deficient dendritic cells.

However, TLR2 was involved in expression of the pro-inflammatory cytokines IL-6 and CXCL1 by DCs following infection with *S. suis* and stimulation by both TLR2 ligands (**Figure S3 in Supplementary Material**). These results demonstrate that though not capable of producing IFN-β following stimulation with control ligands (44), the cells remained responsive to TLR2-dependent *S. suis* stimulation. Surprisingly, TLR4 was partially implicated in IFN-β expression by DCs following infection with both the ST7 strain SC84 and the ST1 strain P1/7 ($p < 0.05$), which corresponded to a reduction of approximately 15%. TLR4 involvement was not observed with the ST25 strain 89-1591 (**Fig. 3B**). A notable difference between the ST1/ST7 and ST25 strains is the absence of SLY in the latter strain. As such, IFN-β expression by wild-type and TLR4$^{-/-}$ DCs was evaluated following infection with the SLY-deficient mutant (**Fig. 3C**). Indeed, the wild-type and SLY-deficient strains induced similar levels of IFN-β by TLR4$^{-/-}$ DCs, indicating that recognition of the SLY by TLR4 contributes to the induction of this cytokine.



Supplementary Figure S3. IL-6 and CXCL1 induced by *S. suis* and bacterial TLR2 ligands partially requires TLR2 expression by dendritic cells. IL-6 and CXCL1 expression by wild-type or TLR2$^{-/-}$ dendritic cells 6 h following infection with *S. suis* ST1 strain P1/7 or activation with the TLR2 ligands PAM3CSK4 (1 μg/mL) and FSL-1 (1 μg/mL). Data represent the mean ± SEM from three independent experiments. ** ($p < 0.01$) and *** ($p < 0.001$) indicate a significant difference between expression by wild-type and TLR2$^{-/-}$ dendritic cells.

Despite the fact that *S. suis* has been described as remaining extracellularly, the largely surface TLR-independence of *S. suis*-induced IFN-β expression by DCs suggested that endosomal TLRs might participate in its induction. In accordance with *S. suis*-induced IFN-β production by DCs being mostly MyD88-dependent, the TLR3, which is MyD88-independent and recruits TRIF, was only minimally implicated, and only following infection with the SLY-negative ST25 strain 89-1591 ($p < 0.05$) (**Fig. 3D**). Meanwhile, both TLR7 and TLR9 were responsible for IFN-β expression by DCs following *S. suis* recognition, regardless of the strain (**Fig. 3D**). Indeed, their absence resulted in a 40 to 60% reduction of IFN-β expression, which was significantly lower when compared with expression by wild-type DCs ($p < 0.01$). In order to evaluate if recognition of *S. suis* by TLR7 and TLR9 was the result of a cooperative effect, DCs dually deficient for TLR7 and TLR9 were created by pretreating cells from either wild-type or $TLR7^{-/-}$ mice with the TLR9 antagonist ODN2088 or its control, ODN2088-Ctrl (**Fig. 3E**). Antagonizing wild-type DCs with ODN2088 resulted in a phenotype similar to that obtained using $TLR9^{-/-}$ DCs. Use of ODN2088-Ctrl on wild-type and $TLR7^{-/-}$ DCs confirmed the specificity of the treatment. When $TLR7^{-/-}$ DCs were antagonized with ODN2088, creating a dual $TLR7^{-/-}/TLR9^{-/-}$ phenotype, a greater decrease, resulting in nearly 80% abrogation of IFN-β expression by wild-type DCs ($p < 0.001$), was observed regardless of the strain. Results of IFN-β by DCs following stimulation with the different purified or synthetic TLR ligands are presented in **Figure S2 in Supplementary Material**.

The interferon regulatory factors 1, 3, and 7 play important though partially redundant roles in IFN-β expression by dendritic cells following *S. suis* infection. Activation of the TLR pathway via the endosomal TLRs usually leads to phosphorylation of IRF3/IRF7 and subsequent production of IFN-β (25). Moreover, though IRF1 is typically associated with induction of type II IFN, crosstalk within the cell and/or simultaneous activation of various cellular pathways may result in IRF1 phosphorylation leading to IFN-β induction (45). Consequently, given that TLR7 and TLR9 are the main TLRs responsible for *S. suis*-induced IFN-β expression by DCs, the expression levels of IRF1, IRF3, and IRF7 in DCs was determined (**Fig. 4A**). All three strains of *S. suis* induced up-regulation of both IRF1 and IRF7, but not IRF3, with no significant differences amongst strains. However, IRF7 expression was significantly more up-regulated than that of IRF1 ($p < 0.05$). Subsequently, in

order to evaluate the role of IRF1, IRF3, and IRF7 in *S. suis*-induced IFN-β expression by DCs, cells isolated from wild-type and knock-out mice infected with the three strains of *S. suis* were evaluated. All three IRFs were significantly implicated in *S. suis*-induced IFN-β expression by DCs ($p < 0.01$), with reductions ranging between 40 and 70% (**Fig. 4B**).

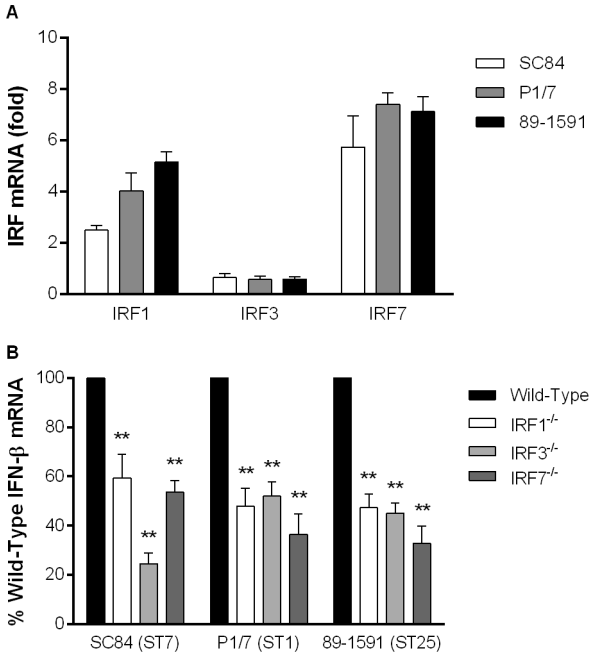


Figure 4. The interferon regulatory factors (IRFs) 1, 3, and 7 are involved in IFN-β expression by dendritic cells (DCs) following infection with *S. suis*. *S. suis*-induced IRF1, IRF3, and IRF7 mRNA expression by DCs 6 h following infection with the different strains (A). IFN-β mRNA expression induced by the different *S. suis* strains following infection of IRF1$^{-/-}$, IRF3$^{-/-}$, or IRF7$^{-/-}$ DCs in comparison with cells from wild-type mice (B). Data represent the mean ± SEM from three or four independent experiments. ** ($p < 0.01$) indicates a significant difference between expression by wild-type and deficient DCs.

***S. suis*-induced IFN-β by dendritic cells requires internalization and phagosome maturation.** Previous studies with group A *Streptococcus* (GAS) and group B *Streptococcus* (GBS) have demonstrated that internalization of the pathogen and maturation of the phagosome are required for IFN-β production by DCs (27, 37). However, and differently from *S. suis*, these two pathogens are well-known to be internalized by phagocytes (46). Given that the endosomal TLR7 and TLR9 are implicated in *S. suis*-induced IFN-β expression by DCs, it

was hypothesized that internalization could be a critical step, even for this classical extracellular pathogen whose CPS protects against phagocytosis (10-12, 47). However, no study has evaluated the capacity of DCs to internalize strains of *S. suis* other than ST1. Consequently, the kinetics of internalization of the three *S. suis* strains by DCs was evaluated. In accordance with previous studies (12), the well-encapsulated ST1 strain P1/7 was poorly internalized, even after 4 h of infection (**Fig. 5A**).

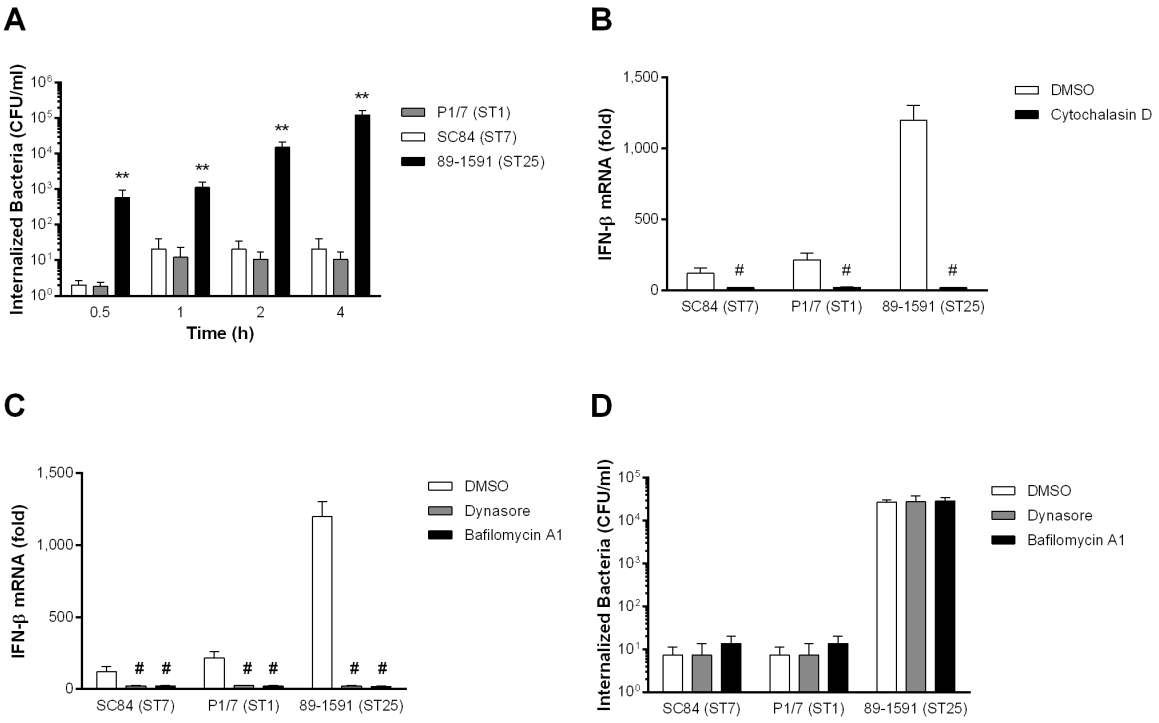
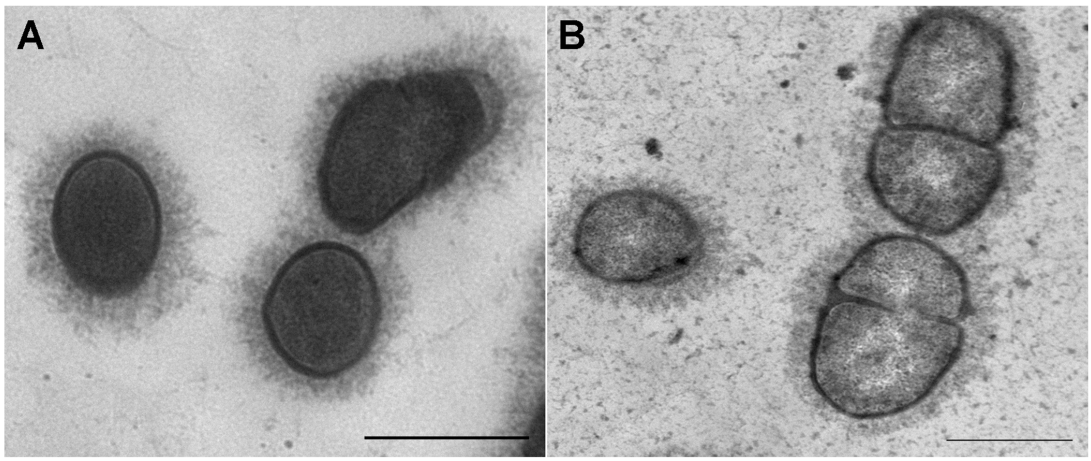


Figure 5. *S. suis*-induced IFN-β expression by dendritic cells requires internalization and phagosome maturation. Internalization kinetics of different *S. suis* strains by dendritic cells (DCs) (A). Implication of actin polymerization (5 µM cytochalasin D) (B), dynamin (8 µM dynasore), and vacuolar-type H^+ ATPase-dependent phagosome acidification (1 µM bafilmoycin A1) (C) on IFN-β mRNA expression by DCs 6 h following infection with *S. suis*. Effect of dynamin remodeling and phagosome acidification on internalization of *S. suis* by DCs 2 h following infection (D). Data represent the mean ± SEM from three independent experiments. ** ($p < 0.05$) indicates a significant difference between 89-1591 and P1/7 or SC84; # ($p < 0.001$) between DCs treated with DMSO (vehicle) and DCs treated with the inhibitors (cytochalasin D, dynasore or bafilomycin A1).

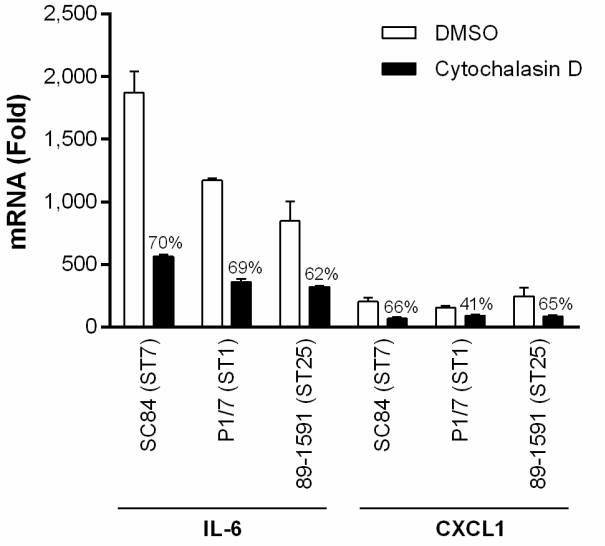
Similar results were obtained with the ST7 strain SC84, for which information was previously unavailable. On the other hand, the intermediate virulent ST25 strain 89-1591, which induces the highest levels of IFN-β, was surprisingly and significantly more internalized by DCs than the ST1 and ST7 strains ($p < 0.01$). Since the CPS is the most important anti-phagocytic factor possessed by *S. suis* serotype 2, these results could have suggested that the strain 89-1591 was less encapsulated. However, it was possible to observe, using transmission electron microscopy, that the ST1 strain P1/7 (**Figure S4A in Supplementary Material**) and the ST25 strain 89-1591 (**Figure S4B in Supplementary Material**) are similarly well-encapsulated, suggesting that the greater internalization of strain 89-1591 by DCs is not the result of a thinner CPS.



Supplementary Figure S4. The intermediate virulent *S. suis* strain 89-1591 is highly encapsulated. Transmission electron micrographs following antibody stabilization of the capsular polysaccharide, using an anti-*S. suis* serotype 2 rabbit serum of ST1 strain P1/7 (*A*) and ST25 strain 89-1591 (*B*). Black bars = 1 μm.

In order to determine if *S. suis* internalization is indeed required for IFN-β expression by DCs, cells were pretreated with cytochalasin D, an inhibitor of actin polymerization, or its vehicle, DMSO. In the absence of actin polymerization, IFN-β expression was completely abrogated ($p < 0.001$) following infection with all three strains of *S. suis* (**Fig. 5B**). In contrast to IFN-β expression, actin polymerization was only partially implicated in DC expression of IL-6 and

CXCL1 following infection with the *S. suis* strain P1/7 (**Figure S5 in Supplementary Material**).



Supplementary Figure S5. *S. suis*-induced IL-6 and CXCL1 expression by dendritic cells is partially internalization-dependent. IL-6 and CXCL1 expression by dendritic cells pretreated with cytochalasin D (5 μM) to inhibit actin polymerization 6 h following infection with *S. suis*. Mock-treated cells (DMSO) were used as controls. Data represent the mean ± SEM from three independent experiments. The percentages of cytochalasin D-mediated inhibition of cytokine expression are indicated.

Once internalized by phagocytes, the pathogen will find itself within the phagosome, which must undergo maturation (48). Amongst the different proteins involved in internalization is the GTPase dynamin, which is required in the case of coated endosomal vesicles (49). As such, dynamin frequently contributes to endosomal signaling of IFN-β (27, 50). Indeed, when inhibiting dynamin activity using the inhibitor dynasore, a near complete abrogation of IFN-β expression ($p < 0.001$) was observed (**Fig. 5C**). However, though dynamin was essential for *S. suis*-induced IFN-β expression by DCs, its role was not internalization-dependent since internalization levels of all three *S. suis* strains did not differ when inhibiting dynamin (**Fig. 5D**).

Following phagosome formation, destruction of the pathogen requires acidification of the compartment, which occurs following fusion with the lysosome (51). This fusion, resulting in the creation of the phagolysosome, leads to acidification of the vesicle in which vacuolar-type H^+ ATPases are implicated (52). Inhibition of these vacuolar-type H^+ ATPases using bafilomycin A1 resulted in a significant ($p < 0.001$) and near complete abrogation of *S. suis*-induced IFN-β expression by DCs (**Fig. 5C**). It should be noted that bafilomycin A1-treatment did not affect internalization of *S. suis* (**Fig. 5D**).

***S. suis* nucleic acids are responsible for inducing IFN-β expression by dendritic cells following endosomal delivery.** The requirement of internalization and phagosome maturation in *S. suis*-induced IFN-β expression by DCs suggests that destruction of the pathogen within the phagolysosome is necessary. TLR7 and TLR9 recognize single-stranded RNA and unmethylated CpG motifs of DNA, respectively (53, 54). To evaluate this hypothesis, bacterial RNA and DNA, isolated from all three *S. suis* strains, were complexed with DOTAP liposomal transfection agent which allows for delivery within the phagosome. As shown in **Fig. 6**, both *S. suis* RNA (**Fig. 6A**) and DNA (**Fig. 6B**) induced significant levels of IFN-β in DCs ($p < 0.001$). Moreover, levels of IFN-β expression were similar between the different *S. suis* strains and between RNA and DNA.

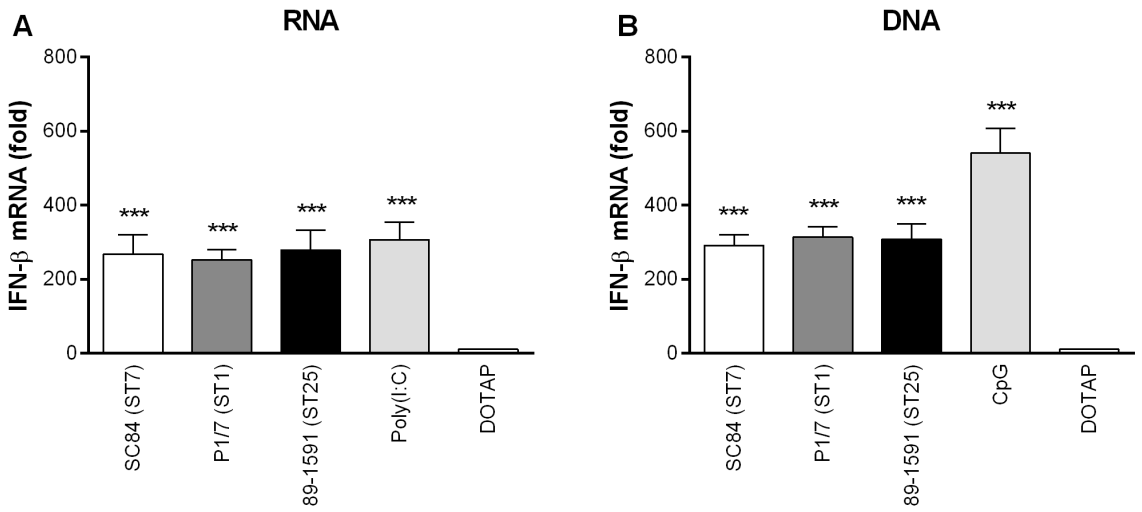


Figure 6. The *S. suis* nucleic acids are responsible for inducing IFN-β expression by dendritic cells following phagosomal delivery. IFN-β mRNA expression by dendritic cells 6 h following transfection with RNA (A) or DNA (B) isolated from the different *S. suis* strains, poly(I:C) or CpG. Nucleic acids were complexed with DOTAP liposomal transfection agent prior to transfection of dendritic cells. Data represent the mean ± SEM from three independent experiments. *** ($p < 0.001$) indicates a significant difference with DOTAP alone (vehicle).

***S. suis*-induced type I interferon by dendritic cells modulates autocrine cytokine production.** Once produced, type I IFN, including IFN-β, will bind to its receptor, IFNAR, located on the surface of most cell types, including DCs (25). Consequently, type I IFN can modulate autocrine production of other inflammatory mediators. As such, the production of certain inflammatory cytokines known to be induced by *S. suis* (20), by DCs derived from wild-type and IFNAR$^{-/-}$ mice, was compared (**Fig. 7**). A significant role of type I IFN ($p < 0.01$) was observed in TNF, IL-6, IL-12p70, and CCL2 (**Fig. 7A-D**) production induced by the ST1 strain P1/7 and the ST25 strain 89-1591, but not by the ST7 strain SC84. On the other hand, no type I IFN-downstream modulation of CCL3 and CXCL1 production was observed for any of the strains tested.

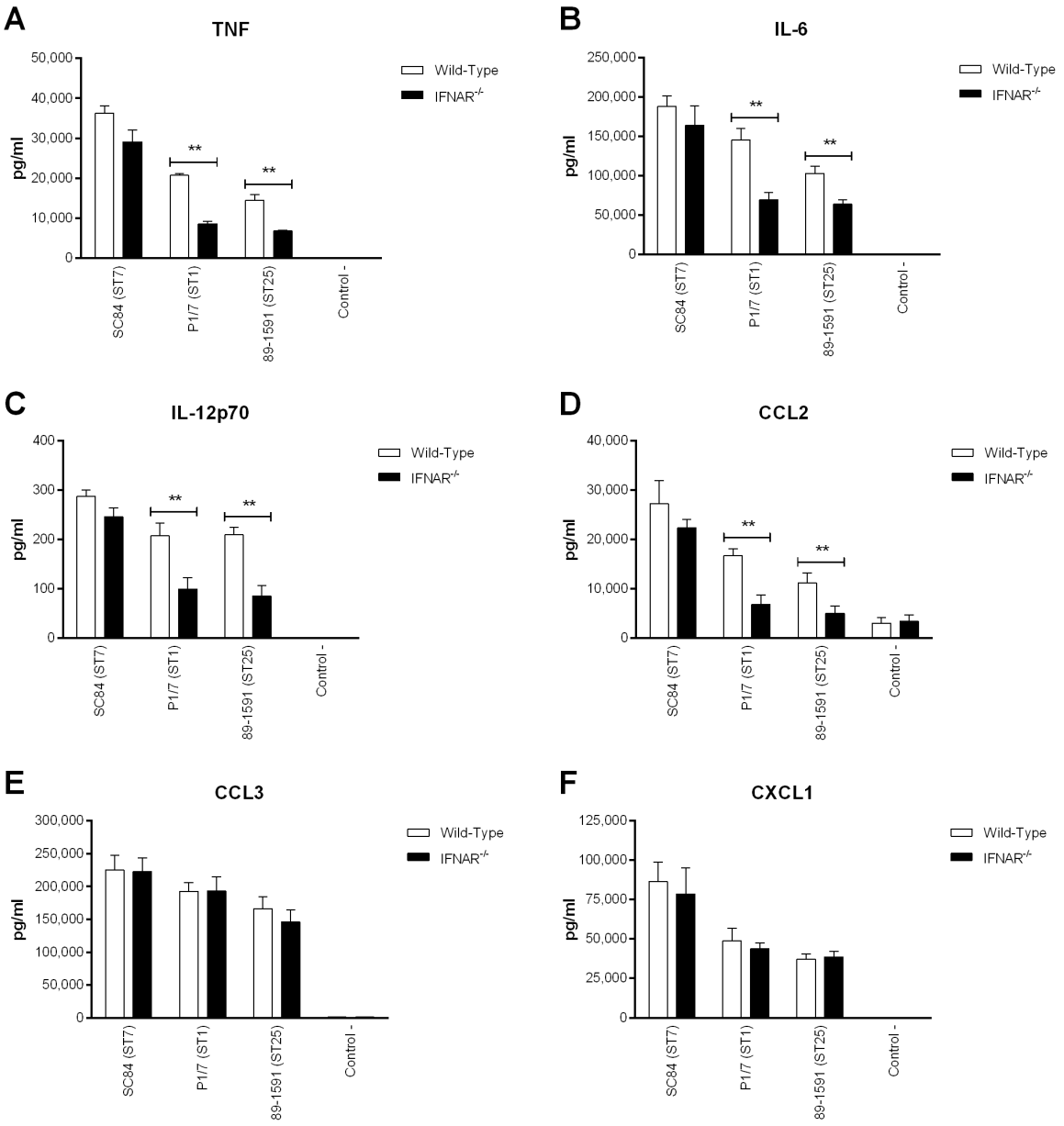


Figure 7. *S. suis*-induced type I interferon produced by dendritic cells modulates autocrine cytokine production. Production of TNF (A), IL-6 (B), IL-12p70 (C), CCL2 (D), CCL3 (E), and CXCL1 (F) by dendritic cells 16 h following infection with the different *S. suis* strains. Data represent the mean ± SEM from four independent experiments. ** ($p < 0.01$) indicates a significant difference in cytokine production between wild-type and IFNAR$^{-/-}$ DCs.

Type I interferon is beneficial for host survival during the *S. suis* serotype 2 systemic infection: Implication in the modulation of systemic inflammation which controls blood bacterial burden. The *in vitro* production of IFN-β by DCs observed in this study, coupled with the up-regulation of this cytokine in mice infected with *S. suis* (3), suggests that IFN-β may be implicated in the balance and/or exacerbation of the systemic inflammation induced by this pathogen, and subsequently, host survival. Consequently, the role of type I IFN during the *S. suis* systemic infection caused by the three strains was evaluated using a well-standardized intraperitoneal C57BL/6 mouse model of infection comparing wild-type and IFNAR$^{-/-}$ mice (38). No role of type I IFN was observed in mouse survival during the systemic infection with the highly virulent ST7 strain SC84, with wild-type or IFNAR$^{-/-}$ mice, with both equally succumbing to the infection (**Fig. 8A**). Meanwhile, type I IFN played a significant role in the survival of mice infected with the virulent ST1 strain P1/7 ($p < 0.05$), with IFNAR$^{-/-}$ mice succumbing at a greater rate than their wild-type counterparts (45% of wild-type mice survived the systemic infection vs. only 10% of the IFNAR$^{-/-}$ mice) (**Fig. 8B**). Interestingly, no role of type I IFN was observed during early (first 72 h p.i.) systemic infection with the intermediate virulent ST25 strain 89-1591 (**Fig. 8C**), which induced high levels of IFN-β *in vitro*. However, given the lower virulence of this strain, which caused only 10 to 20% of mortality at 72 h p.i., this result is not entirely surprising. Less virulent *S. suis* strains are known to cause a delayed mortality, usually by meningitis, as a result of persistent bacteremia (4). As such, survival of mice was evaluated until 14 days p.i. (**Fig. 8D**). Four days p.i., mortality in the wild-type group increased but then remained stable until the end of the experiment. On the other hand, IFNAR$^{-/-}$ mice were significantly more susceptible to the infection than their wild-type counterparts ($p < 0.01$) (**Fig. 8D**). Taken together, these results suggest that type I IFN plays a beneficial role during the *S. suis* infection, at least for the ST25 and, to a certain extent, the ST1 strains, but not with the ST7 strain.

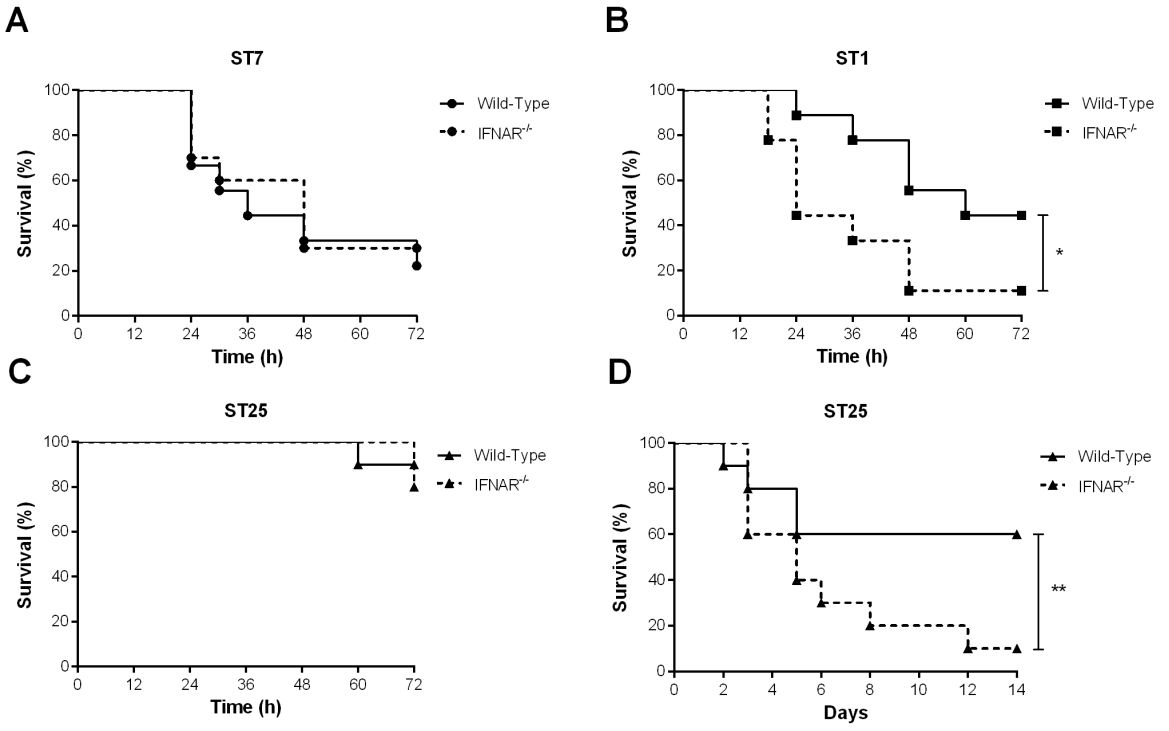


Figure 8. Type I interferon is beneficial for host survival following infection with intermediate virulent and virulent *S. suis* serotype 2 strains. Survival of wild-type and IFNAR$^{-/-}$ mice infected with the different *S. suis* strains: the highly virulent ST7 strain SC84 (A), the virulent ST1 strain P1/7 (B), and the intermediate virulent ST25 strain 89-1591 (C) during the systemic infection (until 72 h post-infection). Survival of wild-type and IFNAR$^{-/-}$ mice infected with strain 89-1591 following both the systemic and central nervous system infections (14 days post-infection) (D). Data represent the survival curves of 15 mice/group. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between survival of wild-type and IFNAR$^{-/-}$ mice.

Host death during the *S. suis* systemic infection is usually the result of uncontrolled blood bacterial burden resulting from excessive bacterial growth, concomitant with an exacerbated systemic inflammatory response (4, 55). As such, the role of type I IFN on aggravated inflammation was evaluated by measuring plasma (systemic) cytokines of both wild-type and IFNAR$^{-/-}$ mice 12 h p.i., as previously described (**Fig. 9**) (4, 38). For TNF, IL-6 , IL-12p70, CCL2, CCL3, and CXCL1 (**Fig. 9A-F**), a significant decrease in the levels produced by IFNAR$^{-/-}$ mice, in comparison to their wild-type counterparts, was only observed for the strains P1/7 and 89-1591 ($p < 0.05$), with the difference being more pronounced in mice infected with the strain 89-1591.

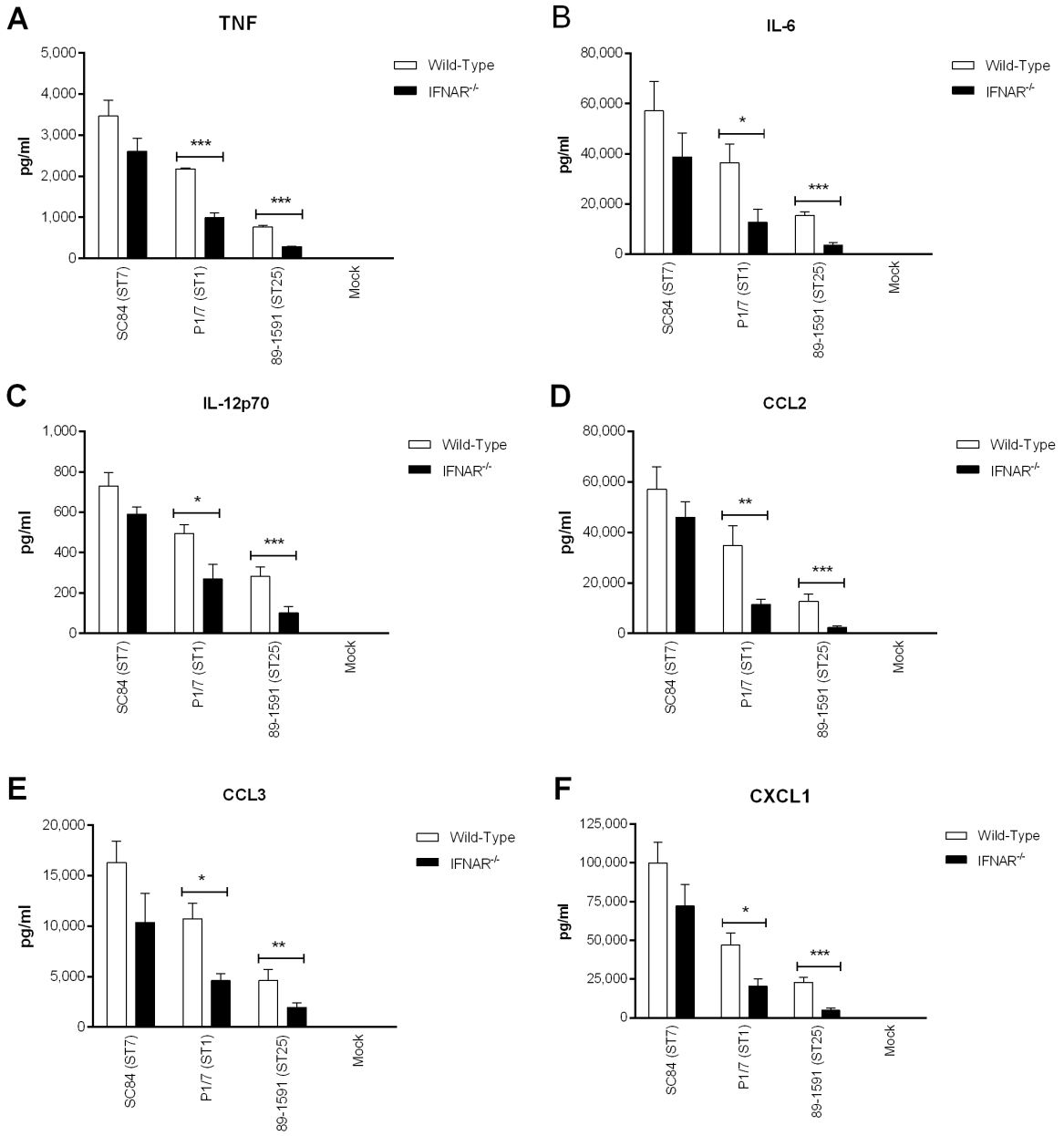


Figure 9. Type I interferon modulates plasma pro-inflammatory cytokines involved in *S. suis*-induced systemic inflammation. Plasma levels of TNF (A), IL-6 (B), IL-12p70 (C), CCL2 (D), CCL3 (E), and CXCL1 (F) in wild-type and IFNAR$^{-/-}$ mice 12 h following infection with the different *S. suis* strains. Data represent the mean ± SEM from 8 mice/group. * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicate a significant difference in plasma levels between wild-type and IFNAR$^{-/-}$ mice.

The second factor responsible for host death during the *S. suis* systemic infection is uncontrolled blood bacterial burden (4). No differences were observed between the acute blood bacterial burden of wild-type and IFNAR$^{-/-}$ mice early after infection (12 h), regardless of the strain (**Fig. 10A**). However, since differences in mortality only became important at later time points, the effect of type I IFN during the *S. suis* infection was possibly not immediate, but rather delayed. Indeed, blood bacterial burden of mice infected with the ST1 strain P1/7 and the ST25 strain 89-1591, but not of those infected with the ST7 strain SC84, was significantly higher in IFNAR$^{-/-}$ mice 48 h p.i. than in their wild-type counterparts ($p < 0.05$) (**Fig. 10B**). Consequently, type I IFN is implicated in the modulation of systemic inflammatory mediators required for control of blood bacterial burden.

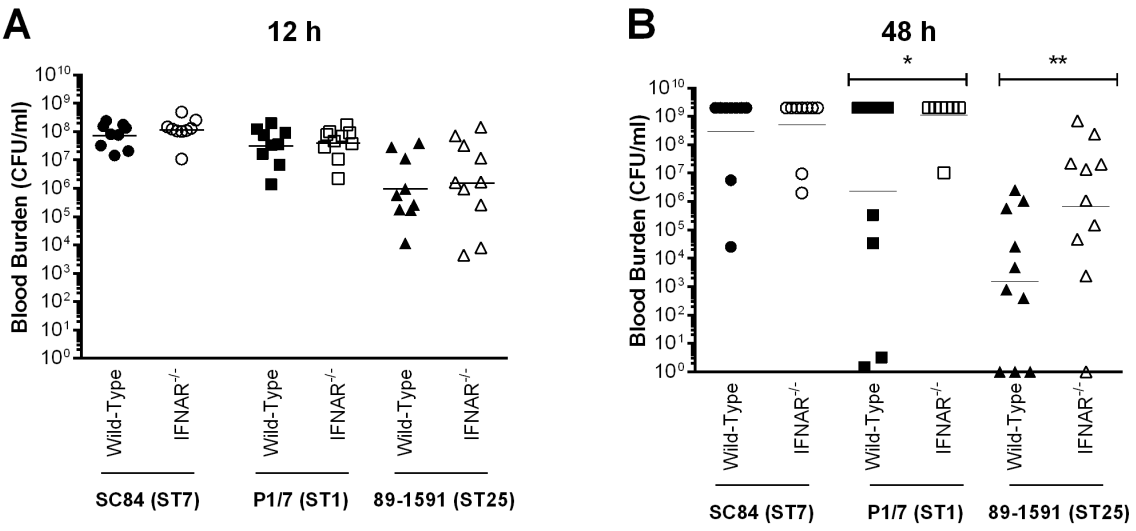


Figure 10. Type I interferon is required for control of blood bacterial burden following infection with intermediate virulent and virulent *S. suis* serotype 2 strains. Blood bacterial burden of wild-type and IFNAR$^{-/-}$ mice infected with the different *S. suis* strains 12 h post-infection (A) or 48 h post-infection (B). Data represent the geometric mean of 15 mice/group. A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to the average burden upon euthanasia, was attributed to euthanized mice. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between blood bacterial burden of wild-type and IFNAR$^{-/-}$ mice.

Discussion

S. suis serotype 2, an important porcine and emerging human pathogen, has always been considered a prototypical extracellular bacterium whose CPS confers important anti-phagocytic properties (6, 18). Consequently, recognition of *S. suis* by the innate immune response was long thought to occur following interaction of bacterial lipoproteins (or other cell wall components) with surface-associated receptors, in particular TLR2 (13, 20, 21, 56). More recently, however, partial implication of the endosomal TLR9 and cytoplasmic nucleotide-binding oligomerization domain-containing protein (NOD) 2 in recognition of *S. suis* by DCs was reported (20), suggesting that *S. suis* or its products might also activate cellular intracellular pathways (in-out signals). Nonetheless, the implications of such intracellular receptors in the pathogenesis of the infection caused by this bacterial pathogen were, so far, unknown.

Since *S. suis* was previously reported to up-regulate levels of IFN-β *in vivo* (3), but not IFN-α, the ability of DCs and macrophages, which are usually important sources of pro-inflammatory mediators, to produce IFN-β following *S. suis* infection was evaluated. In response to *S. suis*, DCs produced higher levels of IFN-β than macrophages, suggesting different activation levels and/or intrinsic differences in cytokine production by both cell types, resulting in part from the activation of varying signaling pathways and cascades (37). Since results obtained with positive controls revealed that DCs and macrophages are both able to induce high IFN-β levels, the differential activation of these two cell types by *S. suis* reflects an intrinsic feature of this pathogen, a characteristic than can be extended to other pathogenic streptococci (27, 37).

Lachance *et al*. suggested that *in vivo* levels of IFN-β were inversely associated with virulence of the strain used (3), a fact that was confirmed in this study with DCs, where the intermediate virulent ST25 strain 89-1591 induced higher levels of IFN-β than the virulent ST1 and ST7 strains. This inverse association is not a trait unique to the strain used, since two other intermediate virulent ST25 strains, as well as an additional ST1 strain (31533), presented results similar to their respective prototypical ST strains. To our knowledge, the inversed

association of virulence with IFN-β induction has not been previously described for other bacterial pathogens. However, this association does not apply to strains of highly virulent to virulent phenotypes, as ST7 and ST1 strains induce similar levels of IFN-β.

The TLR pathway has been traditionally associated with IFN-β induction following recognition by the endosomal TLRs (25), yet, as aforementioned, recognition of *S. suis* has been mostly demonstrated to occur via surface TLRs (13, 20). *S. suis*-induced IFN-β by DCs was MyD88-dependent, indicating that the TLR pathway is almost exclusively implicated in its production. However, even though production of many *S. suis*-induced pro-inflammatory cytokines by DCs has been reported to be mainly TLR2-dependent (20), we were unable to induce TLR2-dependent IFN-β expression by DCs. Though TLR2 activation can result in IFN-β production by macrophages, it was previously suggested that this is not the case for DCs (57, 58). On the other hand, a partial role of TLR4 was observed in IFN-β expression induced by ST1/ST7 strains, but not by ST25 strains: this activation was related to SLY production. This toxin was previously reported to be recognized by TLR4 expressed on peritoneal macrophages (42).

The limited contribution of surface TLRs to *S. suis*-induced IFN-β expression by DCs suggested that endosomal TLRs, of which TLR7 and TLR9 are MyD88-dependent (54), might participate in its induction. The involvement of endosomal TLRs in recognition of *S. suis* has been little evaluated since it has been considered an extracellular pathogen. Consequently, it was unexpected that the TLR7 and TLR9 were equally and primarily responsible for *S. suis*-induced IFN-β. Though TLR7, and to a lesser extent TLR9, were responsible for IFN-β production following recognition of GBS (37), no individual TLR could be identified for GAS (27). Even though *S. suis* and GBS share a similarity in this regard, the pathogenesis of these two encapsulated bacteria greatly differs: the most important difference between these two pathogens is that while *S. suis* is a classical extracellular bacterium protected from phagocytosis by its CPS, well-encapsulated GBS is highly internalized (46). In the case of GBS, IFN-β production by DCs was shown to be completely dependent on IRF1 and only partially IRF7-dependent (37). Interestingly, IRF1, IRF3, and IRF7 were all implicated in *S. suis*-induced IFN-β by DCs, suggesting a partial redundancy in signaling pathways, not

observed for GBS. Indeed, localization of pathogens within the phagosome usually triggers IRF1 and IRF7 (37). Furthermore, IFN-β induced by TLR9 agonists results in IRF1 activation via a phagosome-dependent pathway (59). Participation of IRF3 may be the result of TLR3 or TLR4 activation by SLY-negative and SLY-positive strains, respectively, via a MyD88-independent, TRIF-dependent pathway (45). Feedback loops resulting from crosstalk between pathways could also be responsible for implication of IRF3 (34). In agreement with activation of these intracellular pathways, *S. suis*-induced IFN-β levels inversely correlate with strain-dependent capacity to resist phagocytosis. This hypothesis is supported by the significantly higher levels of IFN-β observed in this study when using the non-encapsulated ST1 mutant strain, which was previously demonstrated to be highly internalized by DCs (20), and by the complete abrogation of IFN-β induction following blockage of actin-dependent internalization. Interestingly, non-encapsulated *S. suis* strains have traditionally been shown to increase cytokine induction by hindering recognition of surface cell wall components, responsible for cell activation by surface-associated receptors (12, 13, 20), mechanism that would not be involved in IFN-β modulation.

Previous studies with GAS and GBS have demonstrated that internalization of the pathogen and maturation of the phagosome are required for IFN-β production by DCs (27, 37). Results obtained in this study demonstrate that though dynamin is required for IFN-β production, this protein is implicated in early pre-acidification steps of phagosome maturation rather than in phagosome formation as evidenced by lack of effect on *S. suis* internalization. Subsequent acidification of the phagosome is required for IFN-β expression by DCs following infection with *S. suis*, suggesting that bacterial processing via hydrolytic degradation is essential for the liberation of TLR7 and TLR9 ligands (27, 37). These results indicate that bacterial nucleic acids were the ligands of TLR7 and TLR9. Indeed, both bacterial RNA, and to a lesser extent, DNA, from GAS and GBS are also responsible for IFN-β production by DCs following stimulation of TLR7 and TLR9 (27, 37). In contrast to GAS and GBS, however, the *S. suis* RNA and DNA induced similar levels of IFN-β, suggesting that both nucleic acids have comparable stimulatory effects. This is supported by the dual implication of TLR7 and TLR9 in *S. suis*-induced IFN-β production by DCs. Nucleic acids isolated from the three *S. suis* strains induced similar levels of IFN-β by DCs, indicating that regardless of virulence, the

different *S. suis* strains possess similar stimulatory properties and differences observed would be mainly attributed to intracellular bacterial levels.

Once produced, type I IFN will bind to its receptor, IFNAR, located on the surface of most cell types, including DCs (25). This autocrine effect modulates *S. suis*-induced pro-inflammatory cytokines by DCs, as well as distal production of cytokines and chemokines by other cells as observed *in vivo* during *S. suis* infection. The IFNAR down-stream effect is complex as evidenced by a lack of effect on DC chemokine production at the single-cell level (60). Yet, when analyzing the global systemic response, the release of the chemokines CCL3 and CXCL1 is indeed modulated by the type I IFN pathway. This pathway was previously demonstrated to amplify TNF signaling following infection with GBS, *S. pneumoniae*, and *E. coli* (28). Pro-inflammatory cytokine signaling is the result of a cascade triggered by TNF, among other mediators, leading to production of IL-6 and IL-12p70 (61). An amplification of these downstream cytokines by type I IFN is thus expected. Similarly, type I IFN was shown to modulate the recruitment of myeloid cells by influencing CCL2 signaling during infection with the intracellular pathogens *Listeria monocytogenes* and *Mycobacterium tuberculosis* (62, 63). Consequently, these results indicate a mechanism complementary to surface-associated receptor activation whereby higher internalization of *S. suis* leads to increased IFN-β induction and subsequent regulation of the pro-inflammatory loop. Our data also suggest that this IFN-β-modulated inflammatory response contributes to control bacterial burden during *S. suis* infection and improves the clinical outcome of infected animals.

It has been previously reported with other pathogens that the induction of IFN-β may be either beneficial or detrimental for the host, as shown using experimental infections. For example, a similar beneficial role was described for GAS (27), GBS (28, 37), and *S. pneumoniae* (28-30). On the other hand, the induction of a strong type I IFN response may also be considered a key factor in early progression of invasive *S. pneumoniae* beyond the lung during development of invasive pneumococcal disease (31). Moreover, type I IFN is associated with suppression of the innate response to certain bacterial infections, such as *L. monocytogenes* and *Francisella tularensis*, resulting in hindered bacterial clearance and deleterious host effects (45).

Taken together, type I IFN is produced by the host following *S. suis* infection and contributes to a regulated inflammatory response. In the case of the intermediate virulent ST25 strain, the elevated IFN-β production modulates systemic pro-inflammatory mediators and appears responsible for the decreased blood bacterial burdens, which ultimately results in a reduction of meningitis and increased host survival. Indeed, it was previously reported that persistent blood bacterial burden is a prerequisite for the development of *S. suis* meningitis (4). Albeit lower levels of IFN-β production, a beneficial effect is also noticed after infection with the virulent ST1 strain. In contrast, type I IFN is unable to counteract the exacerbated inflammatory response and/or bacterial burden induced by the highly virulent ST7 strain. This observation might be related to its genetic particularities, including the presence of a pathogenicity island, and its capacity to induce exaggerated inflammation unparalleled by other *S. suis* strains, resulting in streptococcal toxic shock-like syndrome characterized by a cytokine storm (64-66).

In conclusion, this study demonstrates that, depending on the virulence level of the strain, type I IFN is involved in host defense during the *S. suis* infection by participating in clearance of blood bacterial burden and/or modulation of systemic inflammation. Results also showed that the lower virulence of the North American serotype 2 ST25 strains might be related to a lower resistance to phagocytosis that would lead to increased intracellular receptor activation with consequent IFN-β induction. Underlying mechanisms involved in the control of inflammation and subsequent bacterial burden could help to develop control measures for this important zoonotic infection.

Materials and Methods

Ethics statement

This study was 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 number rech-1570).

Endotoxin-free conditions

Endotoxin (lipopolysaccharide)-free material and solutions were used for bacterial and cell culture work throughout this study.

Streptococcus suis serotype 2 strains and growth conditions

The different well-encapsulated *S. suis* serotype 2 strains, belonging to the most important STs (ST1, ST7, and ST25), and isogenic mutants used in this study are listed in **Table 1**. A highly virulent ST7 strain, isolated during the 2005 human outbreak in China (SC84) (32), a virulent archetypal European ST1 strain (P1/7), and an intermediate virulent North American ST25 strain (89-1591) (3), were used throughout this study. Isogenic mutants were derived from P1/7 (ST1) or a genotypically and phenotypically similar strain, 31533 (ST1), also included in this study. For comparison purposes, two additional intermediate virulent ST25 strains (91-1804 and LPH4) were used in selected experiments. Virulence of the wild-type strains was previously reported (3, 4). *S. suis* strains were grown in Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON, Canada) as previously described (10), diluted in culture medium before experiments, and the number of colony-forming units (CFU)/mL in the final suspension determined by plating on THB agar.

Table 1. *Streptococcus suis* serotype 2 strains used in this study.

Strain	General characteristics	Reference
P1/7	Virulent European ST1 strain isolated from a case of pig meningitis in the United Kingdom	(67)
P1/7Δ*cpsF*	Isogenic non-encapsulated mutant derived from P1/7; in frame deletion of *cpsF* gene	(12)
31533	Virulent European ST1 strain isolated from a case of pig meningitis in France	(68)
31533Δ*sly*	Isogenic suilysin-deficient mutant derived from 31533; in frame deletion of *sly* gene	(69)
31533Δ*dltA*	Isogenic D-alanylation of lipoteichoic acid-deficient mutant derived from 31533; in frame deletion of *dltA* gene	(17)
31533Δ*pgdA*	Isogenic N-deacetylation of peptidoglycan-deficient mutant derived from 31533; in frame deletion of *pgdA* gene	(16)
SC84	Highly virulent ST7 strain isolated from a case of human streptococcal toxic shock-like syndrome during the 2005 outbreak in China	(32)
89-1591	Intermediate virulent North American ST25 strain isolated from a case of pig sepsis in Canada	(70)
91-1804	Intermediate virulent North American ST25 strain isolated from a case of human endocarditis in Canada	(71)
LPH4	Intermediate virulent Asian ST25 strain isolated from a case of human sepsis in Thailand	(72)

Mice

MyD88$^{-/-}$ (B6.129P2(SJL)-*MyD88*$^{tm1.Defr}$/J), TLR2$^{-/-}$ (B6.129-*Tlr2*^{tmKir}/J), TLR3$^{-/-}$ (B6;129S1-*Tlr3*^{tm1Flv}/J), TLR4$^{-/-}$ (B6.B10ScN-*Tlr4*$^{lps-del}$/JthJ), TLR7$^{-/-}$ (B6.129S1-*Tlr7*^{tm1Flv}/J), TLR9$^{-/-}$ (C57BL/6J-*Tlr9*^{M7Btlr}/Mmjax), IRF1$^{-/-}$ (B6.129S2-*Irf1*^{tm1Mak}/J), IRF3$^{-/-}$ (33), IRF7$^{-/-}$ (34), and IFNAR1$^{-/-}$ (B6.129S2-*Ifnar1*^{tm1Agt}/Mmjax) mice on C57BL/6 background were housed under specific pathogen-free conditions alongside their wild-type counterparts (C57BL/6J). Mice were purchased from Jackson Research Laboratories (Bar Harbor, ME, USA), with the exception of IRF3$^{-/-}$ and IRF7$^{-/-}$ mice, which were provided by Dr. K. Mossman.

Generation of bone marrow-derived dendritic cells and macrophages

The femur and tibia of wild-type and knock-out mice were used to generate bone marrow-derived dendritic cells (DCs) and macrophages, as described previously (12, 35). Briefly, hematopoietic bone marrow cells were cultured in RPMI-1640 medium supplemented with 5% (DCs) or 10% (macrophages) heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol (Gibco, Burlington, ON, Canada). Complete medium was complemented with 20% granulocyte macrophage-colony stimulating factor from mouse-transfected Ag8653 cells (35) for DCs or L929 cell-derived macrophage-colony stimulating factor for macrophages (36). Cell purity was at least 85% $CD11c^+$ and $F4/80^+$ cells for DCs and macrophages, respectively (12).

***S. suis* infection of dendritic cells and macrophages**

Cells were resuspended at $1x10^6$ cells/mL in complete medium and stimulated with the different strains of *S. suis* serotype 2 listed in **Table 1** (10^6 CFU/mL; initial multiplicity of infection=1). The conditions used were based on those previously published (12, 20). Cells were harvested in TRIzol (Invitrogen, Burlington, ON, Canada) for mRNA expression 3 h, 6 h or 12 h following infection, and supernatants collected for cytokine measurement 16 h (tumor necrosis factor (TNF), interleukin (IL)-6, IL-12p70, C-C motif chemokine ligand (CCL) 2, CCL3, and C-X-C motif chemokine ligand (CXCL) 1) or 24 h (IFN-β) post-infection (p.i.). Non-infected cells served as negative controls. For neutralization of TLR9, DCs were pretreated with 5 μM ODN2088 (TLR9 inhibitor; InvivoGen, Burlington, ON, Canada) or 5 μM ODN2088-control (Ctrl) for 1 h prior to infection with *S. suis*. Different TLR ligands were used to stimulate cells as positive controls: 1 μg/mL PAM3CSK4 (TLR1/2; InvivoGen), 1 μg/mL FSL-1 (TLR2/6; InvivoGen), 10 μg/mL poly(I:C) (TLR3; Novus Biologicals, Littleton, CO, USA), 100 ng/mL ultrapure *Escherichia coli* lipopolysaccharide (TLR4; InvivoGen), 5 μg/mL imiquimod (TLR7; Novus Biologicals), and 1 μM CpG ODN1826 (TLR9; InvivoGen).

Determination of cell mRNA expression by quantitative RT-PCR (RT-qPCR)

Cell mRNA was extracted according to the manufacturer's instructions (TRIzol) and cDNA generated using the Quantitect cDNA Synthesis Kit (Qiagen, Mississauga, ON, Canada). Real-time qPCR was performed on the CFX-96 Rapid Thermal Cycler System (Bio-Rad, Hercules, CA, USA) using 250 nM of primers (Integrated DNA technologies, Coralville, IA, USA) and SsoFast Evagreen Supermix Kit (Bio-Rad). The cycling conditions were 3 min of polymerase activation at 98°C, followed by 40 cycles at 98°C for 2 sec and 57°C for 5 sec. Melting curves were generated after each run to confirm the presence of a single PCR product. The sequences of primers used in this study are shown in **Supplementary Table 1 (in Supplementary Material)** and were verified to have reaction efficiencies between 90 % and 110 %. The housekeeping genes *Atp5b* and *Gapdh* were determined to be the most stably expressed and used as reference genes to normalize the data. Fold changes in gene expression were calculated using the quantification cycle threshold (Cq) method using the CFX software manager v.3.0 (Bio-Rad). Samples from mock-infected cells served as calibrators.

Supplementary Table S1. Primer sequences used for real-time qPCR.

Gene	Forward (F) and reverse (R) primers
Atp5b	F: ACC AGC CCA CCC TAG CCA CC R: TGC AGG GGC AGG GTC AGT CA
Gapdh	F: CCC GTA GAC AAA ATG GTG AAG R: GAC TGT GCC GTT GAA TTT G
Ifnb	F: CCC AGT GCT GGA GCC ATT GT R: CCC TAT GGA GAT GAC GGA GA
Irf1	F: AGG CAT CCT TGT TGA TGT CC R: AAT TCC AAC CAA ATC CCA GG
Irf3	F:GAT GGC TGA CTT TGG CAT CT R: ACC GGA AAT TCC TCT TCC AG
Irf7	F: AGC ATT GCT GAG GCT CAC TT R: TGA TCC GCA TAA GGT GTA CG
Il6	F: ATG GTA GCT ACC AAA CTG GAT R: TGA AGG ACT CTG GCT TTG TCT
Cxcl1	F: TCT CCG TTA CTT GGG GAC AC R: CCA CAC TCA AGA ATG GTC GC

Cytokine quantification in cell culture supernatants

Levels of IFN-β, TNF, IL-6, IL-12p70, CCL2 (MCP-1), CCL3 (MIP-1α), and CXCL1 (KC) in cell culture supernatants were measured by sandwich ELISA using pair-matched antibodies from BioLegend (Burlington, ON, Canada) for IFN-β and from R&D Systems (Minneapolis, MN, USA) for the other cytokines, according to the manufacturer's recommendations.

Phagocytosis assays

Cells were infected with the different *S. suis* strains and phagocytosis was left to proceed for different times (0.5 to 4 h) at 37°C with 5% CO_2. Multiplicity of infection and assay conditions were chosen based on previous studies regarding the kinetics of *S. suis* phagocytosis by DCs (12). After incubation, penicillin G (5 mg/mL; Sigma-Aldrich, Oakville, ON, Canada) and gentamicin (100 mg/mL; Gibco) were directly added to 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 three times and sterile water added to lyse the cells. Where required, cells were pretreated with either 5 μM cytochalasin D (Santa Cruz Biotech, Dallas, TX, USA), 8 μM dynasore (Sigma-Aldrich), 1 μM bafilomycin A1 (Santa Cruz Biotech) or their vehicle, DMSO (Sigma-Aldrich), for 45 min prior to infection with bacteria, and phagocytosis allowed to proceed for 2 h. The number of CFU recovered per well was determined by plating viable intracellular bacteria on THB agar.

***S. suis* DNA and RNA preparation and transfection of cells**

For bacterial RNA and DNA isolation, bacterial cultures were grown to mid-log phase. Total RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer's instructions, including treatment with DNase I. For DNA preparation, bacteria were harvested in 10 mM Tris, 1 mM EDTA, pH 8.0, and treated with 10% SDS and 20 mg/mL proteinase K (Sigma-Aldrich) for 1 h at 37°C. DNA was isolated using phenol/chloroform/isoamyl alcohol (Sigma-Aldrich) (27). After isolation, bacterial DNA was treated with 10 mg/mL RNase A (Roche, Mississauga, ON, Canada) for 30 min at 37°C. DCs

were transfected with 1 μg of RNA or DNA using DOTAP liposomal transfection agent (Sigma-Aldrich) as described for transfection of bacterial extracts (27, 37).

S. suis serotype 2 mouse model of infection

Six-week-old wild-type C57BL/6 and IFNAR$^{-/-}$ mice were used. Mice were acclimatized to standard laboratory conditions with unlimited access to water and rodent chow (38). These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering, applied throughout this study when animals were seriously affected since mortality was not an endpoint measurement. The different *S. suis* serotype 2 strains, or the vehicle solution (sterile THB), were administered at a dose of $1x10^7$ CFU by intraperitoneal inoculation to groups of 10 to 15 mice for survival and blood bacterial burden. Mice were monitored at least three times daily until 72 h p.i. and twice thereafter until 14 days p.i. Blood bacterial burden was assessed 12 h and 48 h p.i. by collecting 5 μL of blood from the caudal vein, appropriately diluting and plating on THB agar as described above. Blood bacterial burden was also measured prior to euthanasia.

Measurement of plasma (systemic) pro-inflammatory cytokine levels

Eight wild-type and IFNAR$^{-/-}$ mice per group were infected with each strain as described above and the blood collected 12 h p.i. by intracardiac puncture following euthanasia and stabilization with EDTA (Sigma-Aldrich) as previously described (3, 4). Plasma supernatants were collected following centrifugation at 10 000 x *g* for 10 min at 4ºC, and stored at -80ºC. Plasmatic concentrations of TNF, IL-6, IL-12p70, CCL2, CCL3, and CXCL1 were measured using a custom-made cytokine Bio-Plex Pro™ assay (Bio-Rad) according to the manufacturer's instructions. Acquisition was performed on the MAGPIX platform (Luminex®) and data analyzed using the Bio-Plex Manager 6.1 software (Bio-Rad).

Transmission electron microscopy

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich. Bacteria were grown to mid-logarithmic phase and washed in 0.1 M cacodylate buffer, pH 7.3 (Canemco &

Marivac, Canton de Gore, QC, Canada). The CPS was stabilized using specific antibodies as previously described (39). Anti-*S. suis* serotype 2 rabbit serum, produced as previously described (40), was used to gently resuspend bacteria. Next, cells were immobilized in 4% (w/v) agar in 0.1 M cacodylate buffer, pH 7.3. Pre-fixation was performed by 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 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, Hatfield, PA, USA). Thin sections were post-stained with uranyl acetate and lead citrate and examined using a transmission electron microscope at 80 kV (Hitachi model HT7770, Chiyoda, Tokyo, Japan).

Statistical analyses

Normality of data was verified using the Shapiro-Wilk test. Accordingly, parametric (unpaired t-test and one-way ANOVA) or non-parametric tests (Mann-Whitney rank sum test and one-way ANOVA on ranks), where appropriate, were performed to evaluate statistical differences between groups. Log-rank (Mantel-Cox) tests were used to compare survival between wild-type and IFNAR$^{-/-}$ mice. Each test was repeated in at least three independent experiments. $P < 0.05$ and $p < 0.01$ values were considered as statistically significant and highly significant, respectively.

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ANNEXE; ARTICLE XI

Serotype-specific role of antigen I/II in the initial steps of the pathogenesis of the infection caused by *Streptococcus suis*

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Publié dans Veterinary Research. **2017**. 14;48(1):39. doi: 10.1186/s13567-017-0443-4.

Détails sur le rôle du candidat dans la conception de l'article :
Je suis coauteur de l'article. J'ai contribué à la conception et à la construction des vecteurs de complémentation.

Abstract

Streptococcus suis is one of the most important post-weaning porcine bacterial pathogens worldwide. The serotypes 2 and 9 are considered the most virulent and prevalent serotypes involved in swine infections, especially in Europe. However, knowledge of the bacterial factors involved in the first steps of the pathogenesis of the infection remains scarce. In several pathogenic streptococci, expression of multimodal adhesion proteins known as antigen I/II (AgI/II) have been linked with persistence in the upper respiratory tract and the oral cavity, as well as with bacterial dissemination. Herein, we report expression of these immunostimulatory factors by *S. suis* serotype 2 and 9 strains and that AgI/II-encoding genes are carried by integrative and conjugative elements. Using mutagenesis and different *in vitro* assays, we demonstrate that the contribution of AgI/II to the virulence of serotype 2 appears to be modest. In contrast, data demonstrate that the serotype 9 AgI/II participates in self-aggregation, induces salivary glycoprotein 340-related aggregation, contributes to biofilm formation and increased strain resistance to low pH, as well as in bacterial adhesion to extracellular matrix proteins and epithelial cells. Moreover, the use of a porcine infection model revealed that AgI/II contributes to colonization of the upper respiratory tract of pigs. Taken together, these findings suggest that surface exposed AgI/II likely play a key role in the first steps of the pathogenesis of the *S. suis* serotype 9 infection.

Introduction

Streptococcus suis is one of the most important post-weaning bacterial pathogens of pigs and a major economic problem for the porcine industry [1]. Septicemia with sudden death, meningitis, arthritis, and endocarditis are the most frequent clinical signs caused by *S. suis* in pigs [2]. *S. suis* is also a zoonotic agent responsible for numerous human cases of meningitis, septicemia, and streptococcal toxic shock-like syndrome [2]. In Western countries, human *S. suis* infections mostly occur in individuals directly or indirectly linked with the porcine industry. In contrast, the general population is at risk of *S. suis* disease in certain Asian countries where this pathogen has been shown to be an important cause of adult meningitis [3]. Serotype 2 is, globally, considered the most virulent serotype and the one most frequently isolated from both porcine and human infections [4]. The use of multilocus sequence typing has revealed that serotype 2 strains belonging to certain sequence types (STs) are more virulent than others. ST1 strains (virulent) predominate in most Eurasian countries, whereas ST25 and ST28 strains (intermediate and low virulence, respectively) are mainly present in North America [4]. Meanwhile, highly virulent ST7 strains, responsible for at least two important human outbreaks in China, have only been reported in that country [5]. The serotype 9 has recently emerged in certain European countries, such as Spain, the Netherlands, and Germany [4]. Yet, very few studies have addressed the presence of virulence factors in this serotype, and putative virulence factors described for serotype 2 strains may not always be present in serotype 9 strains [6]. Moreover, the first *S. suis* serotype 9 human case of infection was reported in 2015 [7].

The early steps of the pathogenesis of the *S. suis* infection are not well understood [1, 8]. Currently, the most accepted hypothesis is that virulent strains reach the bloodstream after breaching the mucosal epithelium of either the upper respiratory or the gastrointestinal tracts of pigs [1]. Similarly, infection of humans occurs via skin wounds or at the intestinal interface following ingestion of raw or undercooked infected meat [1]. However, the precise mechanisms and virulence factors involved remain unknown. Of note, the upper respiratory tract of pigs, particularly the tonsils and nasal cavities, are important reservoirs of *S. suis* [1]. Furthermore, *S. suis* has also been shown to be present in nearly half of the submaxillary lymph node samples of clinically healthy pigs [9]. Bacterial loads in saliva swab and tonsillar

brush samples are similar, indicating that *S. suis* is indeed a natural inhabitant of the oral cavity [10].

Antigens I/II (AgI/II) have been extensively described in oral as well as in invasive pathogenic streptococci, including *Streptococcus mutans*, *Streptococcus gordonii*, *Streptococcus pyogenes*, and *Streptococcus agalactiae* [11]. AgI/II are immunostimulatory components and multimodal adhesion proteins implicated in host upper respiratory tract and oral cavity persistence and dissemination [11]. Affinity of AgI/II-like proteins for binding salivary glycoproteins, especially the glycoprotein (gp) 340 (also called DMBT1 protein) is a common feature of this protein family [12]. Large quantities of gp340 are present in the saliva of mammals in either a surface-immobilized form or fluid phase form. It is also present at all mucosal surfaces, including the nasal and intestinal cavities [13, 14]. Interestingly, it has been shown that *S. suis* is able to adhere to gp340 and that this protein aggregates certain strains of *S. suis* [15]. However, the strains tested did not express AgI/II when using a heterologous monospecific antibody [15].

In this study, using in silico analyses, genes with homology to those coding for AgI/II were identified in *S. suis*serotype 2 and 9 strains. Using isogenic mutants deficient for the expression and production of AgI/II in both serotype 2 (S2Δ*agI/II*) and serotype 9 (S9Δ*agI/II*), the role of this protein in different aspects of the pathogenesis of the infection caused by *S. suis* was evaluated. We report for the first time that these proteins play a limited or important role in the pathogenesis of the infection caused by *S. suis* serotype 2 and 9, respectively.

Results

Prevalence and molecular characteristics of the *S. suis* AgI/II

Bioinformatics analyses using the *S. suis* (taxid 1307) genome database available in GenBank revealed the presence of genes coding for AgI/II-like proteins in the genomes of serotype 2 strains, including the ST7 strain SC84, ST1 strain BM407, ST25 strain 89–1591, and in a bank of North American *S. suis* serotype 2 ST25 and ST28 strain genomes [18, 19]. However, they were absent from the genome of the reference ST1 strain P1/7. The gene was also present in the genome of the serotype 9 strain D12. Given the low number of

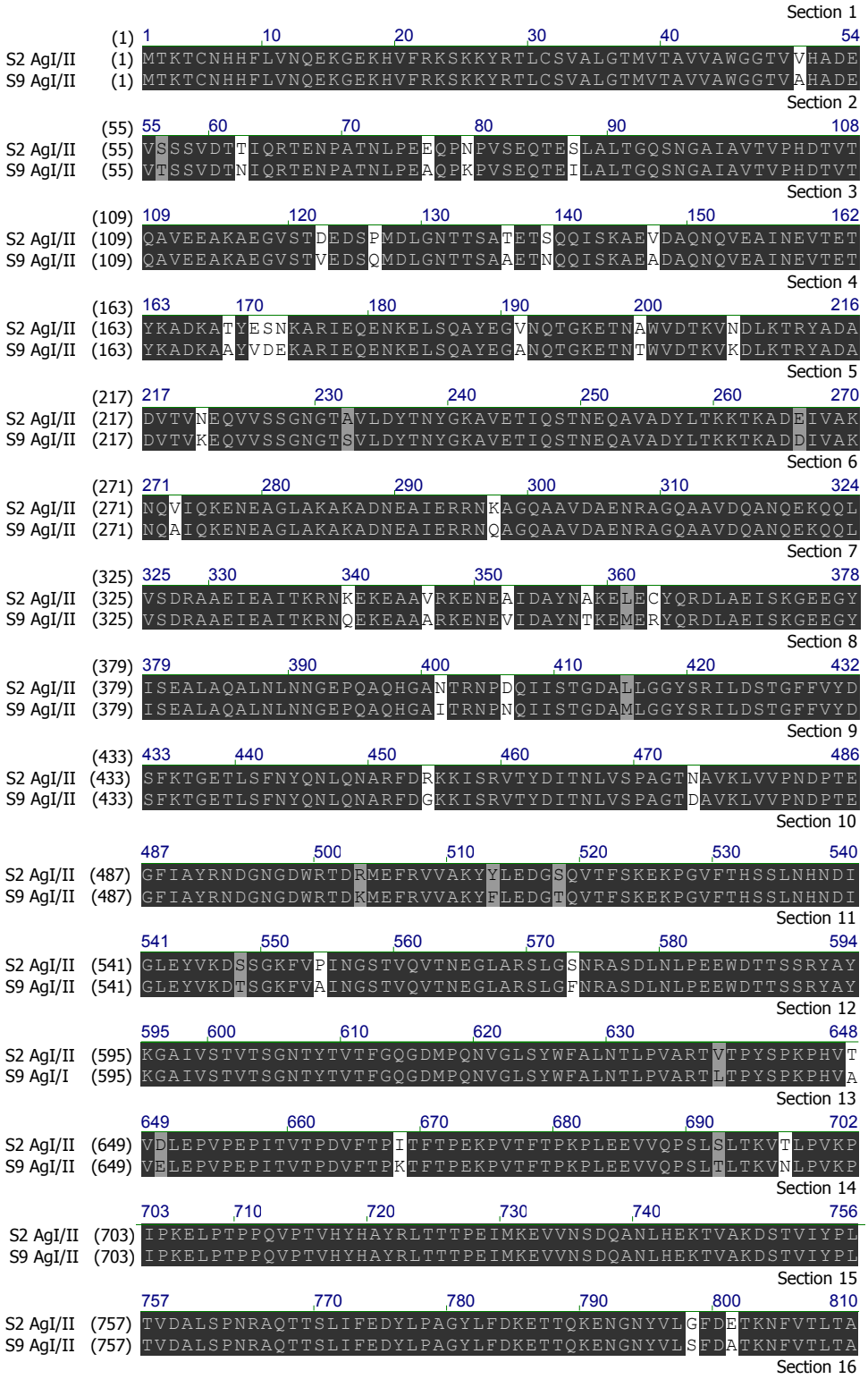
published *S. suis* serotype 9 genomes, PCR analyses were undertaken using field strains, which confirmed the presence of the gene in the 25 strains tested (Supplemental Table S1), including strains from Canada, Germany, and Brazil, as well as in the *S. suis* serotype 9 reference strain from Denmark and a human isolate from Thailand.

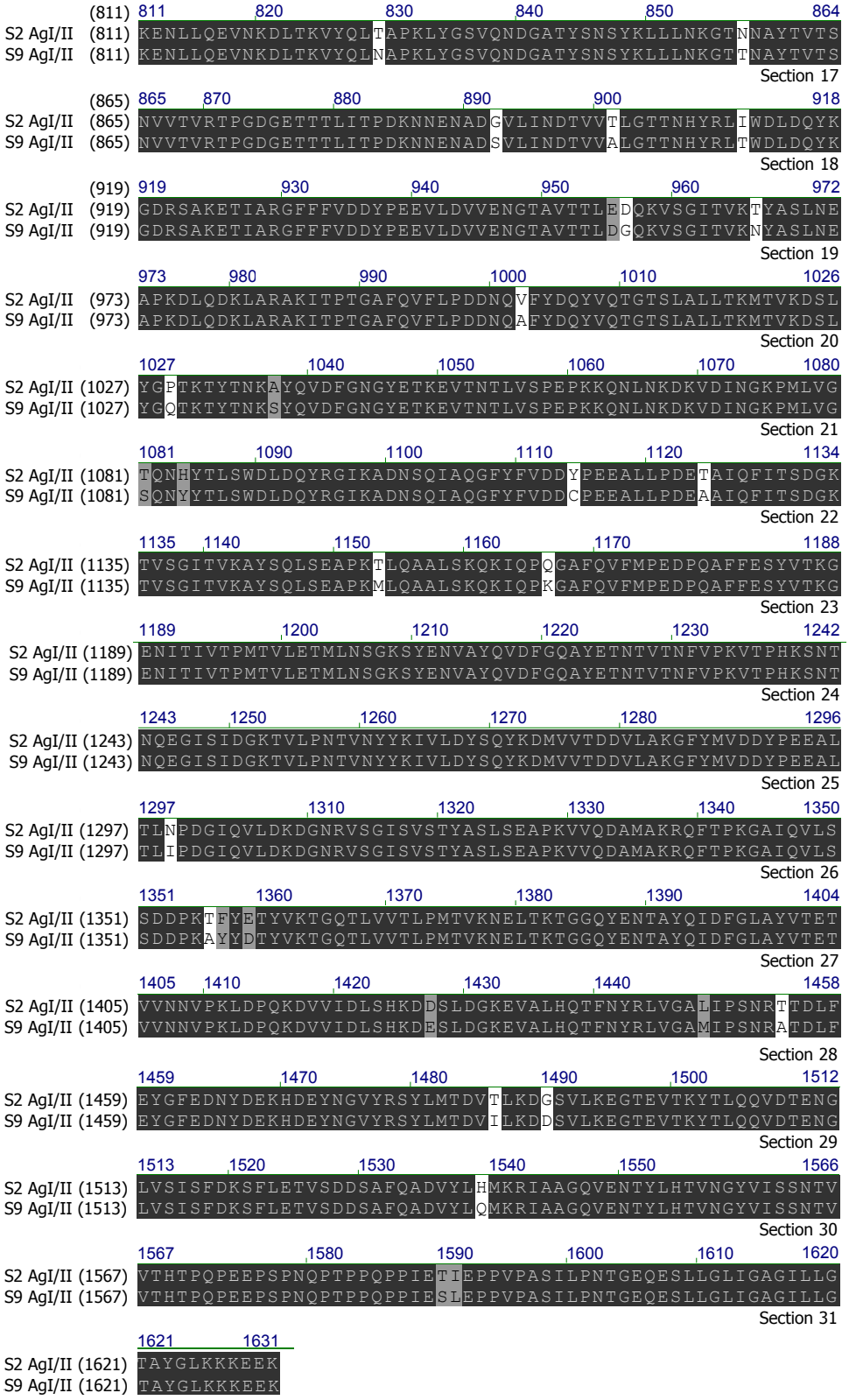
Supplemental Table S1. List of *S. suis* serotype 9 strains used in this study and their characteristics.

Strain	Country	Host Origin	Tissue Origin
1388970	Canada	Pig	Brain
1370475	Canada	Pig	Heart
1509635	Canada	Pig	Brain
1439272	Canada	Pig	Spleen
1406687	Canada	Pig	Lung
1398038	Canada	Pig	Heart
1358915	Canada	Pig	Unknown
1355868	Canada	Pig	Liver
1275845	Canada	Pig	Brain
1273590	Canada	Pig	Lung
1142943	Canada	Pig	Brain
1135776	Canada	Pig	Spleen
1136450	Canada	Pig	Spleen
1137833	Canada	Pig	Spleen
1130349	Canada	Pig	Spleen
1129705	Canada	Pig	Spleen
1092236	Canada	Pig	Kidney
22083	Denmark	Pig	Brain
1580443	Germany	Pig	Blood
1580444	Germany	Pig	Meninges
1580445	Germany	Pig	Unknown
1580446	Germany	Pig	Unknown
1135/10	Brazil	Pig	Brain
1136/10	Brazil	Pig	Spleen
1016/10	Brazil	Pig	Spleen
89-289	Canada	Pig	Brain
1584695	Thailand	Human	Blood

S. suis serotype 2 and 9 genes coding for AgI/II share approximately 95% of nucleotide identity. In addition, the promotors share 92% of nucleotide identity with the −35 and −10 boxes and the ribosome binding site for *agI/II* genes being present in all available genomes. Moreover, the terminators of *agI/II* genes are conserved in all strains (100% of nucleotide identity). The percentage of identity between the AgI/II proteins of serotypes 2 and 9 is 95%, being both highly similar. Alignment of the amino acid sequence of both proteins is

presented in Supplemental Figure S1. Bioinformatics analyses revealed that the *S. suis* AgI/II has a theoretical molecular weight of 180 kDa, which is slightly larger than that of other described AgI/II, probably due to the SspB-like isopeptide-forming domain being repeated thrice in the C-terminal part of the *S. suis* AgI/II (Figure 1) [11]. The *S. suis* AgI/II shares between 29 and 42% of protein sequence identity with other streptococcal AgI/II, such as AspA (*S. pyogenes*), SpaP (*S. mutans*), SspA (*S. gordonii*), and SspB (*S. gordonii*) (Figure 1). Alongside, the *S. suis* AgI/II also shares 32% of protein identity with the aggregation substance PrgB (also called Asc10) of *E. faecalis* [46]. The *S. suis* AgI/II has similar characteristic domains to those described in oral streptococci (Figure 1) [11].


Section 1
(1) 1 10 20 30 40 54
S2 AgI/II (1) MTKTCNHHFLVNQEKGEKHVFRKSKKYRTLCSVALGTMVTAVVAWGGTVVHADE
S9 AgI/II (1) MTKTCNHHFLVNQEKGEKHVFRKSKKYRTLCSVALGTMVTAVVAWGGTVAHADE
Section 2
(55) 55 60 70 80 90 108
S2 AgI/II (55) VSSSVDTTIQRTENPATNLPEEQPNPVSEQTESLALTGQSNGAIAVTVPHDTVT
S9 AgI/II (55) VTSSVDTNIQRTENPATNLPEAQPKPVSEQTEILALTGQSNGAIAVTVPHDTVT
Section 3
(109) 109 120 130 140 150 162
S2 AgI/II (109) QAVEEAKAEGVSTDEDSPMDLGNTTSATETSQQISKAEVDAQNQVEAINEVTET
S9 AgI/II (109) QAVEEAKAEGVSTVEDSQMDLGNTTSAAETNQQISKAEADAQNQVEAINEVTET
Section 4
(163) 163 170 180 190 200 216
S2 AgI/II (163) YKADKATYESNKARIEQENKELSQAYEGVNQTGKETNAWVDTKVNDLKTRYADA
S9 AgI/II (163) YKADKAAYVDEKARIEQENKELSQAYEGANQTGKETNTWVDTKVKDLKTRYADA
Section 5
(217) 217 230 240 250 260 270
S2 AgI/II (217) DVTVNEQVVSSGNGTAVLDYTNYGKAVETIQSTNEQAVADYLTKKTKADEIVAK
S9 AgI/II (217) DVTVKEQVVSSGNGTSVLDYTNYGKAVETIQSTNEQAVADYLTKKTKADDIVAK
Section 6
(271) 271 280 290 300 310 324
S2 AgI/II (271) NQVIQKENEAGLAKAKADNEAIERRNKAGQAAVDAENRAGQAAVDQANQEKQQL
S9 AgI/II (271) NQAIQKENEAGLAKAKADNEAIERRNQAGQAAVDAENRAGQAAVDQANQEKQQL
Section 7
(325) 325 330 340 350 360 378
S2 AgI/II (325) VSDRAAEIEAITKRNKEKEAAVRKENEAIDAYNAKELECYQRDLAEISKGEEGY
S9 AgI/II (325) VSDRAAEIEAITKRNQEKEAAARKENEVIDAYNTKEMERYQRDLAEISKGEEGY
Section 8
(379) 379 390 400 410 420 432
S2 AgI/II (379) ISEALAQALNLNNGEPQAQHGANTRNPDQIISTGDALLGGYSRILDSTGFFVYD
S9 AgI/II (379) ISEALAQALNLNNGEPQAQHGAITRNPNQIISTGDAMLGGYSRILDSTGFFVYD
Section 9
(433) 433 440 450 460 470 486
S2 AgI/II (433) SFKTGETLSFNYQNLQNARFDRKKISRVTYDITNLVSPAGTNAVKLVVPNDPTE
S9 AgI/II (433) SFKTGETLSFNYQNLQNARFDGKKISRVTYDITNLVSPAGTDAVKLVVPNDPTE
Section 10
487 500 510 520 530 540
S2 AgI/II (487) GFIAYRNDGNGDWRTDRMEFRVVAKYYLEDGSQVTFSKEKPGVFTHSSLNHNDI
S9 AgI/II (487) GFIAYRNDGNGDWRTDKMEFRVVAKYFLEDGTQVTFSKEKPGVFTHSSLNHNDI
Section 11
541 550 560 570 580 594
S2 AgI/II (541) GLEYVKDSSGKFVPINGSTVQVTNEGLARSLGSNRASDLNLPEEWDTTSSRYAY
S9 AgI/II (541) GLEYVKDTSGKFVAINGSTVQVTNEGLARSLGFNRASDLNLPEEWDTTSSRYAY
Section 12
595 600 610 620 630 648
S2 AgI/II (595) KGAIVSTVTSGNTYTVTFGQGDMPQNVGLSYWFALNTLPVARTVTPYSPKPHVT
S9 AgI/ (595) KGAIVSTVTSGNTYTVTFGQGDMPQNVGLSYWFALNTLPVARTLTPYSPKPHVA
Section 13
649 660 670 680 690 702
S2 AgI/II (649) VDLEPVPEPITVTPDVFTPITFTPEKPVTFTPKPLEEVVQPSLSLTKVTLPVKP
S9 AgI/II (649) VELEPVPEPITVTPDVFTPKTFTPEKPVTFTPKPLEEVVQPSLTLTKVNLPVKP
Section 14
703 710 720 730 740 756
S2 AgI/II (703) IPKELPTPPQVPTVHYHAYRLTTTPEIMKEVVNSDQANLHEKTVAKDSTVIYPL
S9 AgI/II (703) IPKELPTPPQVPTVHYHAYRLTTTPEIMKEVVNSDQANLHEKTVAKDSTVIYPL
Section 15
757 770 780 790 800 810
S2 AgI/II (757) TVDALSPNRAQTTSLIFEDYLPAGYLFDKETTQKENGNYVLGFDETKNFVTLTA
S9 AgI/II (757) TVDALSPNRAQTTSLIFEDYLPAGYLFDKETTQKENGNYVLSFDATKNFVTLTA
Section 16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S2 AgI/II (811) KENLLQEVNKDLTKVYQLTAPKLYGSVQNDGATYSNSYKLLLNKGTNNAYTVTS
S9 AgI/II (811) KENLLQEVNKDLTKVYQLNAPKLYGSVQNDGATYSNSYKLLLNKGTTNAYTVTS
Section 17
(865) 865 870 880 890 900 918
S2 AgI/II (865) NVVTVRTPGDGETTTLITPDKNNENADGVLINDTVVTLGTTNHYRLIWDLDQYK
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Section 18
(919) 919 930 940 950 960 972
S2 AgI/II (919) GDRSAKETIARGFFFVDDYPEEVLDVVENGTAVTTLEDQKVSGITVKTYASLNE
S9 AgI/II (919) GDRSAKETIARGFFFVDDYPEEVLDVVENGTAVTTLDGQKVSGITVKNYASLNE
Section 19
973 980 990 1000 1010 1026
S2 AgI/II (973) APKDLQDKLARAKITPTGAFQVFLPDDNQVFYDQYVQTGTSLALLTKMTVKDSL
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1027 1040 1050 1060 1070 1080
S2 AgI/II (1027) YGPTKTYTNKAYQVDFGNGYETKEVTNTLVSPEPKKQNLNKDKVDINGKPMLVG
S9 AgI/II (1027) YGQTKTYTNKSYQVDFGNGYETKEVTNTLVSPEPKKQNLNKDKVDINGKPMLVG
Section 21
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S2 AgI/II (1081) TQNHYTLSWDLDQYRGIKADNSQIAQGFYFVDDYPEEALLPDETAIQFITSDGK
S9 AgI/II (1081) SQNYYTLSWDLDQYRGIKADNSQIAQGFYFVDDCPEEALLPDEAAIQFITSDGK
Section 22
1135 1140 1150 1160 1170 1188
S2 AgI/II (1135) TVSGITVKAYSQLSEAPKTLQAALSKQKIQPQGAFQVFMPEDPQAFFESYVTKG
S9 AgI/II (1135) TVSGITVKAYSQLSEAPKMLQAALSKQKIQPKGAFQVFMPEDPQAFFESYVTKG
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Section 24
1243 1250 1260 1270 1280 1296
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Section 25
1297 1310 1320 1330 1340 1350
S2 AgI/II (1297) TLNPDGIQVLDKDGNRVSGISVSTYASLSEAPKVVQDAMAKRQFTPKGAIQVLS
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Section 27
1405 1410 1420 1430 1440 1458
S2 AgI/II (1405) VVNNVPKLDPQKDVVIDLSHKDDSLDGKEVALHQTFNYRLVGALIPSNRTTDLF
S9 AgI/II (1405) VVNNVPKLDPQKDVVIDLSHKDESLDGKEVALHQTFNYRLVGAMIPSNRATDLF
Section 28
1459 1470 1480 1490 1500 1512
S2 AgI/II (1459) EYGFEDNYDEKHDEYNGVYRSYLMTDVTLKDGSVLKEGTEVTKYTLQQVDTENG
S9 AgI/II (1459) EYGFEDNYDEKHDEYNGVYRSYLMTDVILKDDSVLKEGTEVTKYTLQQVDTENG
Section 29
1513 1520 1530 1540 1550 1566
S2 AgI/II (1513) LVSISFDKSFLETVSDDSAFQADVYLHMKRIAAGQVENTYLHTVNGYVISSNTV
S9 AgI/II (1513) LVSISFDKSFLETVSDDSAFQADVYLQMKRIAAGQVENTYLHTVNGYVISSNTV
Section 30
1567 1580 1590 1600 1610 1620
S2 AgI/II (1567) VTHTPQPEEPSPNQPTPPQPPIETIEPPVPASILPNTGEQESLLGLIGAGILLG
S9 AgI/II (1567) VTHTPQPEEPSPNQPTPPQPPIESLEPPVPASILPNTGEQESLLGLIGAGILLG
Section 31
1621 1631
S2 AgI/II (1621) TAYGLKKKEEK
S9 AgI/II (1621) TAYGLKKKEEK

Supplemental figure S1. *S. suis* serotype 2 (S2) and serotype 9 (S9) AgI/II amino acid sequence alignment. Alignment was performed using Vector NTI 11.5. Conserved amino acids appear in light gray and identical amino acids in dark gray.

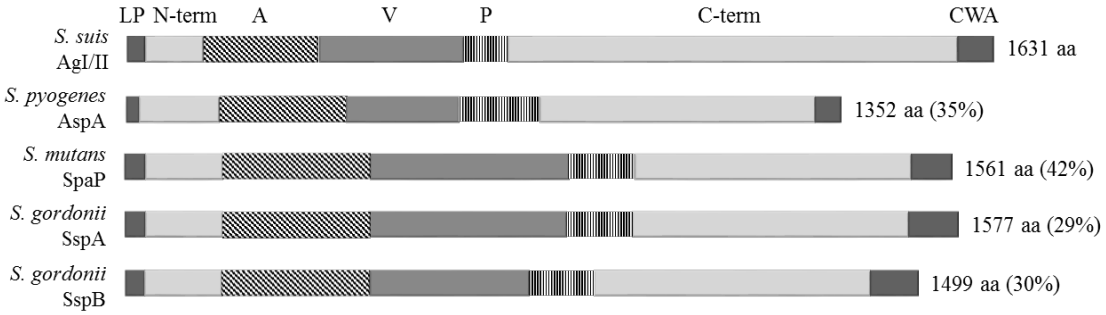


Figure 1. Characteristics of AgI/II proteins present in different streptococci. The leader peptide signal (LP), N-terminal domain (N-term), alanine-rich region (A), variable region (V), proline-rich region (P), and C-terminal domain, and the cell wall anchorage domain (CWA) containing the LPXTG domain are illustrated for the *S. suis* AgI/II, *S. pyogenes* AspA, *S. mutans* SpaP, and *S. gordonii* SspA and SspB. Amino acid (aa) size and percentage of *S. suis* AgI/II protein identity are also indicated.

Further bioinformatics investigations, including the use of the ICEberg database, revealed that the gene encoding for the AgI/II protein in the serotype 2 strain SC84 is carried by the 89 K ICE (89 Kbp) [47], while that of the serotype 2 ST1 strain BM407 is carried by two putative ICEs annotated as ICE*Ssu*(BM407)1 and ICE*Ssu*(BM407)2) (75 and 80 Kbp, respectively). Moreover, the gene coding for AgI/II in the serotype 9 strain D12 is also carried by an element sharing 95% of nucleotide identity with the whole sequence of ICE*Ssu*(BM407)1. Altogether, these analyses suggest that the *S. suis* AgI/II are mainly carried by ICEs.

Confirmation of AgI/II-deficient mutants in both *S. suis* serotypes 2 and 9

Production of AgI/II by the serotype 2 and 9 strains SC84 and 1135776, respectively, was confirmed by immunoblotting using mono-specific antisera produced with the recombinant protein, rAgI/II (Figure 2). The proteins had a molecular weight of approximately

180 kDa, as predicted by bioinformatics analyses. Deletion of the *agI/II* gene resulted in absence of detectable signal while complementation of the mutant strains restored detection with a band at the expected molecular weight (Figure 2). Growth of the S2Δ*agI/II* and S9Δ*agI/II* mutants as well as that of the complemented strains was similar to their respective wild-type strains (data not shown).

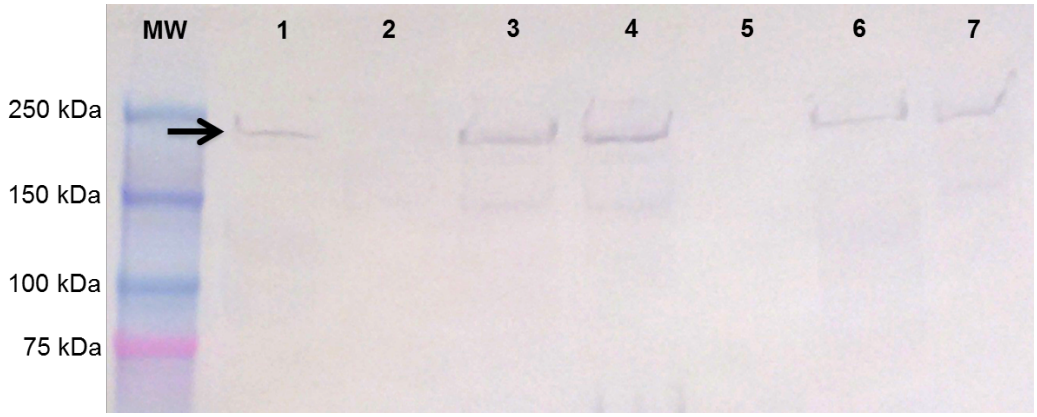
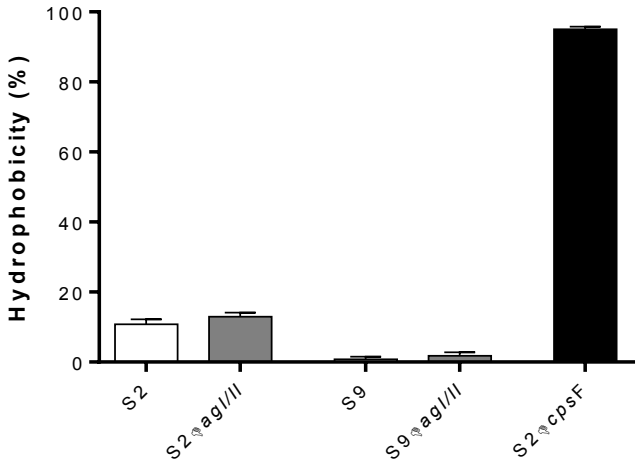


Figure 2. The AgI/II protein is expressed in the *S. suis* serotype 2 and 9 wild-type strains but is absent in S2Δ*agI/II* and S9Δ*agI/II* mutant strains. Western blot using cell wall extracts from *S. suis* serotype 2 (wells 1 to 3) and serotype 9 (wells 4 to 6): serotype 2 wild-type strain SC84 (well 1) and serotype 9 wild-type strain 1135776 (well 4); mutant strains S2Δ*agI/II* (well 2) and S9Δ*agI/II* (well 5); and complemented strains S2CΔ*agI/II* (well 3) and S9CΔ*agI/II* (well 6). Expected bands at approximately 180 kDa, shown by the arrow, were observed for the serotype 2 and 9 wild-type and complemented strains, similar to that obtained with the purified AgI/II protein (well 7), used as a positive control. MW: Molecular weight makers.

It was previously described that AgI/II positively impacts surface hydrophobicity of oral streptococci. However, we did not observe significant differences in hydrophobicity between the *S. suis* serotype 2 or 9 wild-type strains and their AgI/II-deficient mutants (S2Δ*agI/II* and S9Δ*agI/II*) (Supplemental Figure S2). Interestingly, the serotype 2 wild-type strain was significantly more hydrophobic than that of serotype 9 ($p < 0.05$).



Supplemental Figure S2. Hydrophobicity of the *S. suis* serotype 2 (S2) and serotype 9 (S9) wild-type and AgI/II-deficient mutant strains. Hydrophobicity was determined using n-hexadecane and the non-encapsulated *S. suis* strain S2Δ*cpsF* included as a positive control. Data represent the mean ± SEM from at least three independent experiments.

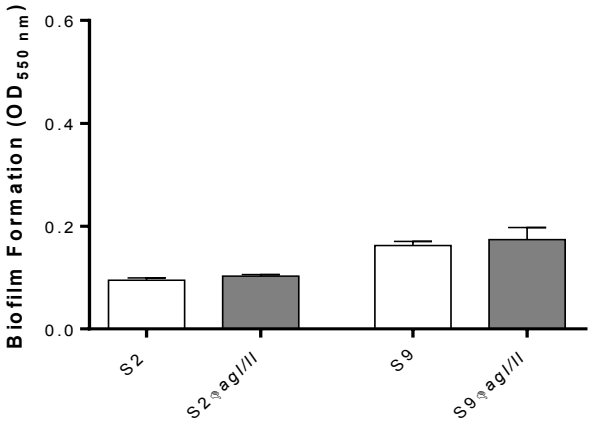
In vitro pathogenesis assays

Serotype-dependent role of the *S. suis* AgI/II in self-aggregation and biofilm formation

S. suis serotype 2 self-aggregation was not modified by the absence of AgI/II (Figure 3A). However, deletion of AgI/II significantly reduced self-aggregation of *S. suis* serotype 9 by 80% ($p < 0.01$) (Figure 3A). On the other hand, self-aggregation was completely restored when using the complemented S9CΔ*agI/II* strain (Figure 3A). Thus, the serotype 9 AgI/II, but not that of serotype 2, is involved in bacterial self-aggregation.

The role of AgI/II in biofilm formation was evaluated for both serotype 2 and 9 in the presence of porcine fibrinogen. The capacity of the serotype 2 strain to form biofilm was relatively low, and no difference was observed in the absence of AgI/II (Figure 3B). On the other hand, the serotype 9 wild-type strain showed a significantly greater capacity to form biofilm than the wild-type serotype 2 strain in the presence of porcine

fibrinogen ($p < 0.01$). Furthermore, the serotype 9 AgI/II was significantly involved in this bacterial function ($p < 0.001$) (Figure 3B). The capacity to form biofilm was restored in the complemented S9CΔ*agI/II* strain (Figure 3B). Minimal biofilm formation was observed in the absence of porcine fibrinogen for both the serotype 2 and 9 strains (Supplemental Figure S3). Consequently, the serotype 9 AgI/II, but not that of serotype 2, plays an important role in the capacity to form biofilm.



Supplemental Figure S3. Biofilm formation by the *S. suis* serotype 2 (S2) and serotype 9 (S9) wild-type and AgI/II-deficient mutant strains in the absence of porcine fibrinogen. Biofilm formation capacity was quantified after 24 h of incubation at 37°C in the absence of porcine fibrinogen. Data represent the mean ± SEM from at least three independent experiments.

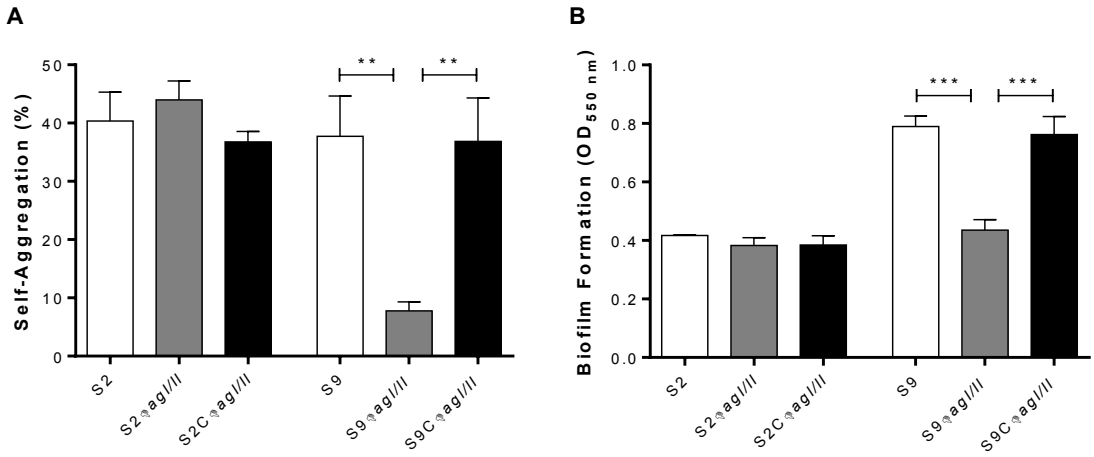


Figure 3. The *S. suis* serotype 9 (S9) AgI/II, but not that of the serotype 2 (S2), is implicated in bacterial self-aggregation and biofilm formation. The role of the *S. suis* AgI/II was evaluated with regards to cell-to-cell aggregation in fluid phase (A) and biofilm formation capacity in the presence of porcine fibrinogen (B) after 24 h of incubation at 37°C. Data represent the mean ± SEM from at least

three independent experiments. ** ($p < 0.01$) and *** ($p < 0.001$) indicate a significant difference between the *S. suis* S9 wild-type or complemented strain (S9CΔ*agI/II*) and AgI/II-deficient mutant (S9Δ*agI/II*).

The *S. suis* AgI/II increases both porcine salivary agglutinin induced-aggregation and adhesion to salivary agglutinins

Salivary agglutinins are major receptors of streptococcal AgI/II [12]. Thus, we investigated the interactions of the *S. suis* serotype 2 and 9 AgI/II with fluid phase (miming the conditions in saliva) and surface-immobilized (miming mucosa such as in the oral cavity) pSAGs. pSAGs collected from pig saliva was obtained at a concentration of 50 μg/mL, which is similar to that usually obtained for human salivary agglutinins [37].

Results showed a significantly more rapid and greater aggregation of both *S. suis* serotype 2 or serotype 9 strains in the presence of pSAGs ($p < 0.05$) (Figure 4). Moreover, this fluid phase pSAG-induced aggregation significantly increased with time ($p < 0.05$) (Figure 4). However, the pSAG-mediated aggregation induced by the serotype 9 strain was significantly higher than that induced by the serotype 2 strain, but only after 60 min of incubation ($p < 0.05$) (Figure 4). AgI/II-deficiency significantly reduced fluid phase pSAG-induced aggregation for both serotypes ($p < 0.05$) (Figures 5A and B), and complementation of AgI/II-deficient mutants restored fluid phase pSAG-induced aggregation ($p < 0.01$) (Figures 5A and B).

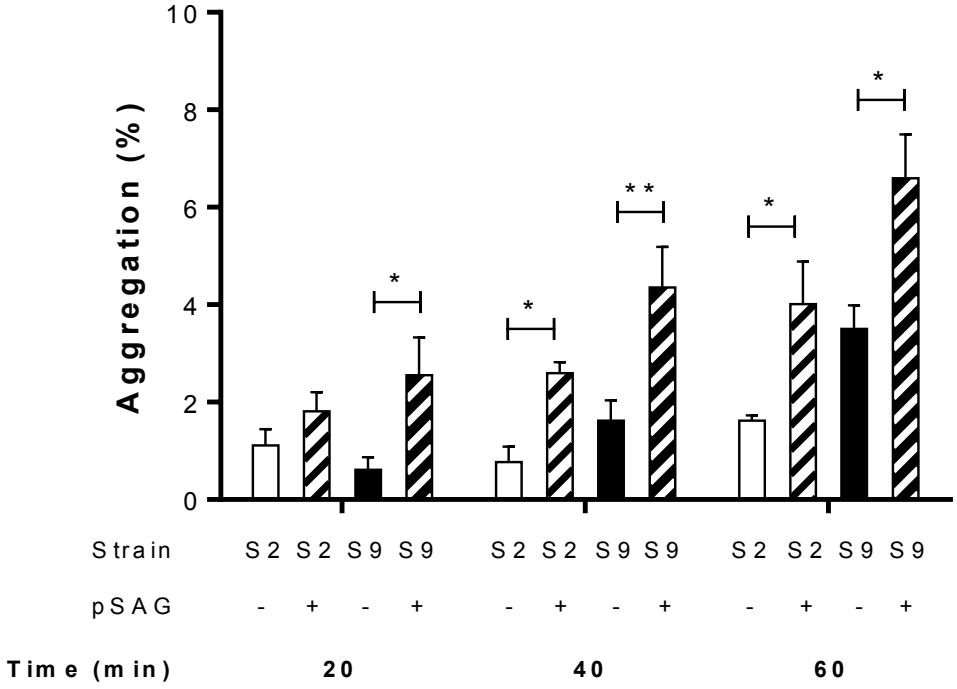


Figure 4. Porcine salivary agglutinins (pSAG) aggregate *S. suis* serotype 2 (S2) and serotype 9 (S9). Evaluation of the fluid phase aggregation in the absence (-) or presence (+) of pSAG. Aggregation in the absence of pSAG reflects self-aggregation only. Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference of *S. suis* S2 or S9 aggregation in the absence and presence of pSAG.

The adhesion of *S. suis* to surface-immobilized pSAGs was then evaluated using ELISA. Since background obtained with crude pSAGs was very elevated (data not shown), the gp340-derived peptide SRCRP2, described as the major binding sequence for AgI/II [37], was used. Results showed that deletion of the *S. suis* serotype 2 *agI/II* had no effect on adhesion to SRCRP2 (Figure 5C), while that of serotype 9 significantly reduced adhesion to SRCRP2 ($p < 0.05$), but only at a concentration of 200 µg/mL (Figure 5D). As expected, complementation of the *S. suis* serotype 9 AgI/II-deficient mutant restored adhesion to SRCRP2 (Figure 5D).

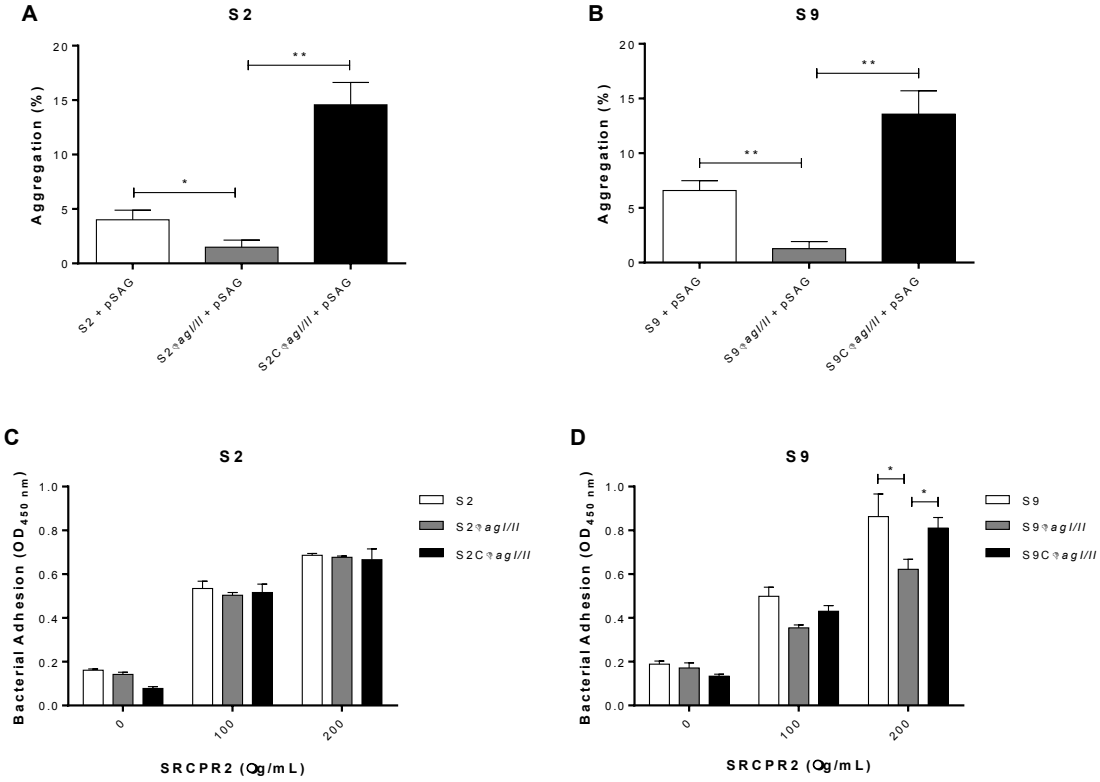


Figure 5. The *S. suis* serotype 2 (S2) and serotype 9 (S9) AgI/II are involved in adhesion to fluid phase porcine salivary agglutinins (pSAG), but only for S9 with surface-immobilized pSAG. Evaluation of the fluid phase aggregation of S2 (A) and S9 (B) strains to pSAG or to surface-immobilized gp340-derived peptide SRCRP2 by S2 (C) and S9 (D), the latter being measured by ELISA. Data represent the mean ± SEM format least three independent experiments. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between the *S. suis* S2 or S9 wild-type or complemented strain (S2CΔ*agI/II* or S9CΔ*agI/II*) and the AgI/II-deficient mutants (S2Δ*agI/II* or S9Δ*agI/II*).

Taken together, these results demonstrate that AgI/II promotes pSAG-induced aggregation when in fluid phase for both serotypes, and adhesion to the gp340-derived peptide SRCRP2 at a high concentration for serotype 9 only.

The *S. suis* AgI/II confers protection to acid stress

Once swallowed, *S. suis* will reach the stomach, in which it must overcome hostile environmental conditions such as low pH. We thus investigated the role of AgI/II and aggregation in resistance to low pH. Acid stress killing assays revealed that the *S. suis* serotype 2 AgI/II was not involved in acid resistance at pH 3 (Figure 6A) nor at pH 5 (Figure 6C). On the other hand, results showed that the S9Δ*agI/II* mutant strain survived significantly less than its wild-type strain ($p < 0.05$) at both pH 3 (Figure 6B) and pH 5 (Figure 6D). Thus, AgI/II confers partial protection to *S. suis* serotype 9, but not to serotype 2, against acidic environments.

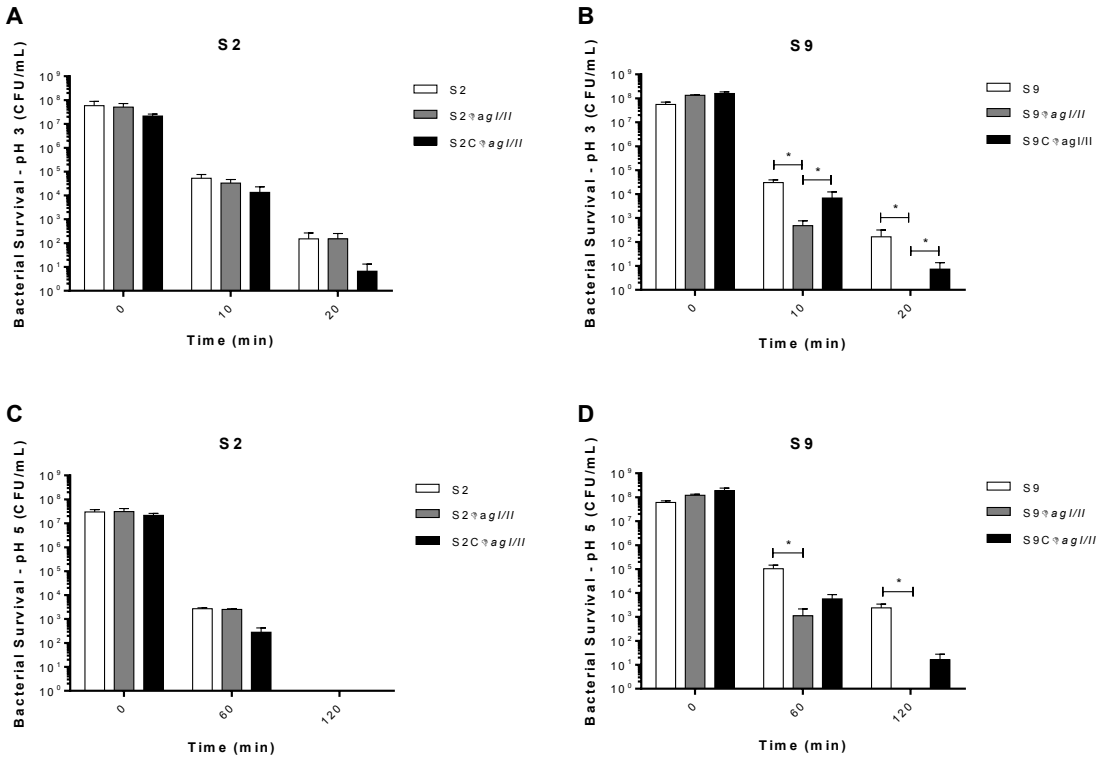


Figure 6. The *S. suis* serotype 9 (S9) AgI/II, but not that of serotype 2 (S2), is involved in protection against acid stress. Effect of acid stress on *S. suis* S2 and S9 viability, determined at pH 3 **(A & B)** and pH 5 **(C & D)**. Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) indicates a significant difference between the *S. suis* S9 wild-type or complemented strain (S9CΔ*agI/II*) and AgI/II-deficient mutant (S9Δ*agI/II*).

The *S. suis* serotype 9 AgI/II contributes to adhesion to extracellular matrix proteins and to porcine epithelial cells

AgI/II was previously described in other streptococci as binding ECM proteins and contributing to adhesion to and invasion of epithelial cells. Our results showed that while the serotype 2 AgI/II was not involved in adhesion to collagen I, that of the serotype 9 played a significant role ($p < 0.01$) (Figures 7A and B). In accordance, complementation of the S9Δ*agI/II* mutant restored the wild-type phenotype (Figure 7B). Moreover, as previously described with other serotype 2 strains [38], the serotype 2 wild-type strain used in this study (SC84) did not bind porcine fibrinogen (Figure 7C). On the other hand, the serotype 9 wild-type strain did bind to porcine fibrinogen, with absence of AgI/II significantly reducing this ability ($p < 0.05$) (Figure 7D). Once again, complementation of the S9Δ*agI/II* mutant strain restored this adhesion capacity (Figure 7D). Finally, the deletion of the *S. suis* serotype 9 *agI/II* gene and, to a lesser extent, that of the serotype 2, significantly decreased adhesion to plasma fibronectin ($p < 0.05$) (Figures 7E and F). Consequently, these results demonstrate the importance of AgI/II as a multimodal adhesin for *S. suis* serotype 9 while only playing a minor role for serotype 2.

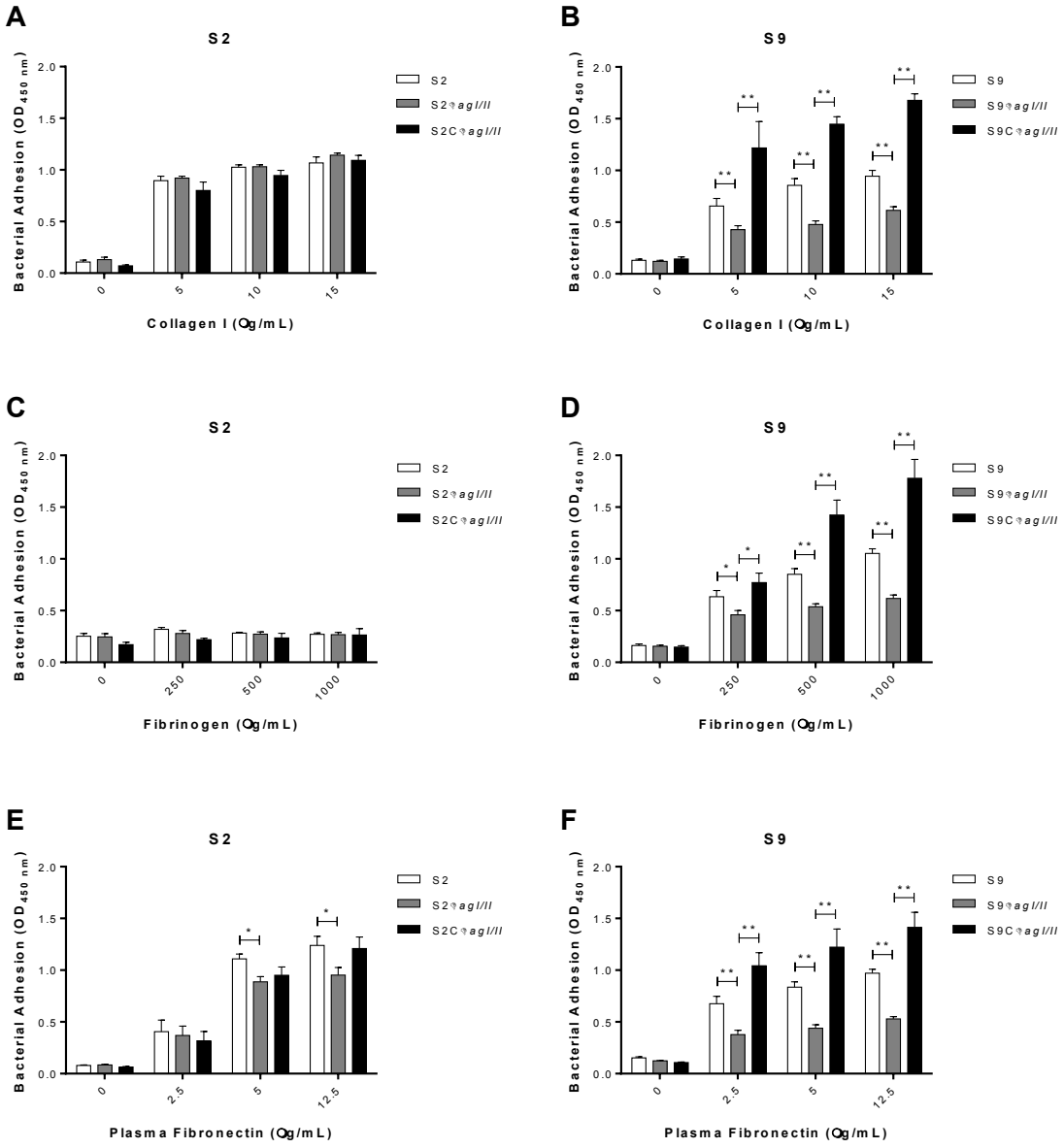


Figure 7. The *S. suis* serotype 9 (S9) AgI/II and, to a lesser extent, that of serotype 2 (S2), are bacterial adhesins for extracellular matrix proteins. Adhesion of the *S. suis* S2 and S9 strains to different concentrations of collagen I (A & B), fibrinogen (C & D), and plasma fibronectin (E & F) as evaluated by ELISA. Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between the wild-type or complemented strain (CΔ*agI/II*) and the AgI/II-deficient mutant (Δ*agI/II*).

The role of AgI/II in adhesion to and invasion of porcine tracheal epithelial cells was subsequently investigated. Interestingly, the serotype 9 wild-type strain adhered significantly more to epithelial cells than did the serotype 2 ($p < 0.05$) (Figure 8). Adhesion assays revealed a significant decrease in adhesion to epithelial cells in the absence of AgI/II for the serotype 9 ($p < 0.05$), equivalent to 30% of wild-type strain adhesion, with complementation restoring adhesion (Figure 8). On the other hand, no differences were observed between the *S. suis* serotype 2 wild-type strain and its AgI/II-deficient mutant (Figure 8). Low levels of epithelial cell invasion were observed for both serotypes, with no role of AgI/II being evident (data not shown). Taken together, these results reveal that AgI/II is implicated in adhesion to host proteins and epithelial cells for serotype 9 and, to a lesser extent, for serotype 2.

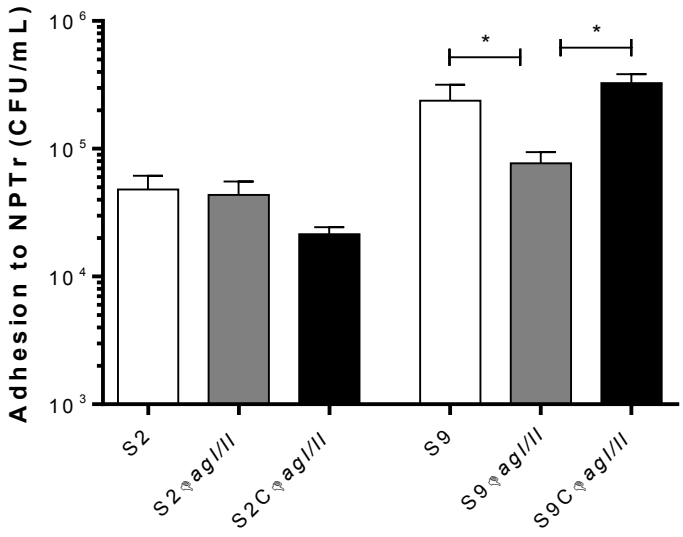


Figure 8. The *S. suis* serotype 9 (S9) AgI/II, but not that of the serotype 2 (S2), is involved in adhesion to porcine tracheal epithelial cells. Adhesion of the *S. suis* S2 and S9 strains to NPTr cells after 2 h of incubation with a multiplicity of infection of 10. Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) indicates a significant difference between the *S. suis* S9 wild-type or complemented strain (S9CΔ*agI/II*) and the AgI/II-deficient mutant (S9Δ*agI/II*).

Role of AgI/II in colonization of the oral and nasal cavities of pigs

Given that in vitro results demonstrated an important role of AgI/II for *S. suis* serotype 9, we next evaluated the contribution of this protein in colonization using a porcine infection model. Animals were divided into two groups and infected with either the serotype 9 wild-type strain or the AgI/II-deficient mutant by intranasal inoculation. Evaluation of serotype 9 colonization revealed that the number of wild-type strain recovered from the nasal cavities significantly increased over time until day 12 post-infection (p.i.) ($p < 0.05$), whereas the number of S9Δ*agI/II* remained stable throughout the experiment (Figure 9A). Moreover, AgI/II-deficient mutants were recovered in significantly lower numbers from the nasal cavities of pigs on days 5, 8, and 12 p.i. ($p < 0.05$) (Figure 9A). Although the number of serotype 9 wild-type strain and AgI/II-deficient mutant in the nasal cavities of pigs was similar 21 days p.i. (Figure 9A), AgI/II-deficiency resulted in significantly reduced colonization of tonsils ($p < 0.05$) (Figure 9B). Together, these results strongly suggest that the serotype 9 AgI/II contributes to colonization of the porcine respiratory tract.

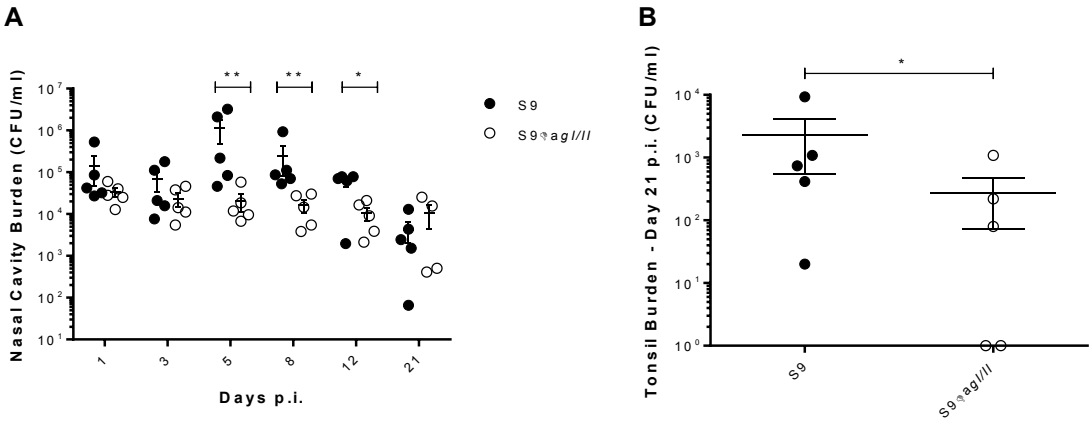


Figure 9. The *S. suis* serotype 9 (S9) AgI/II is implicated in colonization of the porcine respiratory tract. An intranasal porcine model of infection was used to determine the implication of the *S. suis* S9 AgI/II in colonization of the nasal cavity (A) and tonsils 21 days post-infection (B). Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between presence of the *S. suis* S9 wild-type strain and the AgI/II-deficient mutant (S9Δ*agI/II*).

Discussion

AgI/II proteins have been extensively described in oral pathogenic streptococci as multimodal adhesion proteins and immunostimulatory components implicated in host upper respiratory tract and oral cavity persistence and dissemination [11]. In addition, it has been shown that AgI/II proteins potentially play multiple roles in *Streptococcus* adherence, colonization, and microbial community development [11]. These proteins have also been described in pyogenic streptococci, such as *S. pyogenes* and *S. agalactiae,* but they have never been identified in *Streptococcus pneumoniae* [11]. An initial goal of this study was to determine whether *S. suis* possesses these putative virulence factors. We showed that most of the *S. suis* serotype 2 available genomes, including from different STs, possess genes encoding AgI/II. Interestingly, the gene was absent from the ST1 strain P1/7, which is commonly used as a reference for investigation of virulence [6]. We also identified AgI/II-encoding genes in the genome of the Chinese serotype 9 strain D12, in the serotype 9 reference strain 22083, as well as in a collection of 25 serotype 9 field strains (added herein given the limited number of serotype 9 genomes available), alongside a human isolate, tested by PCR.

It is widely recognized that mobile genetic elements such as insertion sequences, transposons, bacteriophages, plasmids, and genomic islands are key drivers of genomic evolution and bacterial adaptation. Among them, ICEs are chromosomal genetic elements that play an important role in horizontal gene transfer [48]. In both *S. pyogenes* and *S. agalactiae*, AgI/II are encoded by genes carried by ICEs, which can spread not only to other *S. pyogenes* and *S. agalactiae* strains, but also to other streptococci [49, 50]. Meanwhile, different ICEs have been described in *S. suis* [51], of which the 89 K ICE carried by the *S. suis* serotype 2 strain SC84 has been suggested to be responsible, at least in part, for the higher virulence of this strain [52]. Interestingly, results obtained in this study showed that the *S. suis agI/II* genes are mainly carried by ICEs. As such, it may be suggested that acquisition of AgI/II by *S. suis* occurred via horizontal transfer following acquisition of ICEs.

Persistence of *S. suis* in the oral cavity may contribute to the pathogenesis of the infection. Our data showed that AgI/II plays an important role in self-aggregation for *S. suis* serotype 9. This role was even more important in the presence of salivary glycoproteins,

such as gp340. It has been previously shown that human salivary gp340 was able to aggregate an untypeable, a serotype 1, and a serotype 2 *S. suis* strain [15]. However, these strains were negative for the expression of AgI/II as evaluated by immunoblot using a polyclonal antibody raised against the *S. mutans* proteins [15]. In the present study, we showed that purified soluble pSAGs increase the ability of *S. suis* to aggregate and that AgI/II played an important role in such interactions for serotype 9 and, to a lesser extent, serotype 2. Fluid phase and surface-immobilized gp340 expose different binding properties and, consequently, differentially recognize adhesive phenotypes of diverse bacterial species. Herein, we showed that AgI/II also played a role in the *S. suis* serotype 9 adhesion to the surface-immobilized gp340-derived peptide SRCRP2. Similarly, the AgI/II from *S. suis* serotype 9 also played an important role in biofilm formation.

The relationship between the saliva-dependent aggregation, attachment to salivary glycoproteins, and biofilm formation in the oral cavity and pathogenesis of the infection caused by pathogenic streptococci is not very clear. On the one hand, aggregation (clumping) may presumably allow "bacterial clearance" from the oral cavity via swallowing [53]. It is usually accepted that the main route of infection for pigs is through the respiratory tract. However, more recently, the oral route (as clearly described in humans) has also been suggested as a portal of entry in pigs [54]. Although a recent report showed that disease could not be induced in an experimental infection by the oral route in post-weaned animals [55], a role of early colonization of the intestine of pre-weaned piglets followed by direct invasion through intestinal epithelial cells in animals under post-weaned stress could not be completely ruled out [1]. In the present study, an increased susceptibility to low pH (usually found in the stomach) was observed for *S. suis* serotype 9 in the absence of the *agI/II* gene. As such, it may be hypothesized that AgI/II induces bacterial self-mediated and salivary agglutinin-mediated aggregation and biofilm formation for serotype 9, which would increase, at certain moments, the swallowing of large amounts of bacteria. AgI/II would subsequently increase bacterial protection against the low pH of the stomach, thus allowing colonization of the intestine. However, this hypothesis remains to be confirmed.

It has been proposed that adhesion to epithelial cells is one of the most important initial steps of the pathogenesis of the infection caused by *S. suis* [1]. Similarly to other pathogens, *S. suis* is also able to bind ECM components, which have been suggested to be implicated as cell receptors [1]. At least 28 different *S. suis*components have been described to be involved in such interactions so far [1, 6]. In the present study, it was clearly shown that the AgI/II plays an important role in the adhesion of *S. suis* serotype 9 to collagen I, fibrinogen, and fibronectin. In the case of serotype 2, this protein plays a minimal role in adhesion to fibronectin and none to collagen I. As previously described, the serotype 2 strain was unable to bind fibrinogen [38]. The lack of binding to the latter may also explain differences observed in biofilm formation (in the presence of this protein) between serotype 2 and serotype 9 strains and the important role played by the serotype 9 AgI/II.

The implication of AgI/II in the adhesion to epithelial cells was further evaluated using porcine tracheal epithelial cells as a model [40]. Firstly, it was interesting to note that the serotype 9 wild-type strain presented higher adhesion levels than the serotype 2 strain, a fact that has been previously reported with other porcine cells [54]. A role was attributed to AgI/II in the adhesion of serotype 9 since a significant reduction of adhesion to these cells was observed using the S9Δ*agI/II* mutant. This reduction of adhesion could be explained by a reduction in the interactions with ECM components (as described above) or through a direct effect of the AgI/II as an adhesin. In fact, this protein has been described to be directly involved in epithelial cell adhesion and invasion by *S. gordonii* through β1 integrin recognition [56]. Using a different mechanism, this protein was also involved in adhesion/invasion of *S. pyogenes* to these cells [56].

Previous studies showed that the *S. pyogenes* AgI/II is implicated in upper respiratory tract colonization [57]. Since results showed that AgI/II plays important roles in vitro for serotype 9, its implication in colonization of the upper respiratory tract was investigated in pigs. As previously described, pigs infected by the serotype 9 wild-type strain and its isogenic S9Δ*agI/II* mutant via the intranasal route did not develop clinical signs of infection [43]. However, a slight, yet significantly lower colonization of the upper respiratory tract by the mutant strain, and, subsequently at the tonsillar level, was observed, suggesting that this

protein may collaborate in bacterial colonization during the first steps of the infection. However, additional studies should be carried out to confirm this hypothesis.

In conclusion, the presence of AgI/II is herein reported for the first time in *S. suis*. This protein appears to play important or limited roles during the first steps of the pathogenesis of the infection caused by serotypes 9 and 2, respectively. Since the gene and protein sequences are highly similar between both serotypes, the observed differences are more difficult to explain than anticipated, and several hypotheses may be proposed. Firstly, a particular motif specific to the gene coding for the serotype 9 AgI/II might be responsible for the phenotypic differences highlighted in this study. Secondly, the *S. suis* serotype 2 and 9 *agI/II* genes are both carried by ICEs, which vary, creating differing genetic contexts and, consequently, differential gene regulation. Thirdly, critical *S. suis* virulence factors still remain poorly known [6]; the lack of a dominant role of the serotype 2 AgI/II observed herein might also be due to compensation by other virulence factors that result in bacterial redundancy [6]. Further studies are presently underway to explore these avenues. Overall, AgI/II may contribute to the colonization of the upper respiratory tract of pigs and could represent important surface bacterial components implicated in the first steps of the pathogenesis of the infection caused by *S. suis*.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The virulent serotype 2 ST7 strain SC84, responsible for the 2005 human outbreak in China [5], and the serotype 9 strain 1135776 (isolated from a diseased pig in Canada) were used herein as models to study the role of Ag I/II in the pathogenesis of the infection caused by *S. suis*. Twenty-five additional *S. suis* serotype 9 strains recovered from diseased pigs were also used to evaluate the prevalence of *agI/II* genes by PCR (Supplemental Table S1). Seventeen of these strains originated from Canada, 3 from Brazil, 1 from Denmark (reference strain), and 4 from Germany. A strain isolated from a human case of infection was also included [7]. The *S. mutans* strain Ingbritt was used as a tool for collection of porcine salivary agglutinins (pSAGs) whereas the *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA), MC1061 [16], and BL21(DE3) (Invitrogen) strains were used for DNA manipulations and/or AgI/II protein production. The different *Streptococcus* and *E. coli* strains were grown at 37 °C in Todd Hewitt (THB) under static conditions or in Luria-Bertani broth (Becton Dickinson, Franklin Lakes, NJ, USA) with shaking, respectively. Antibiotics (Sigma-Aldrich, St-Louis, MO, USA), where needed, were used at the following concentrations for *S. suis* and *E. coli*: spectinomycin at 500 and 50 µg/mL and erythromycin at 5 and 200 µg/mL, respectively. Ampicillin was also used at a concentration of 50 µg/mL for *E. coli*.

Table 1. Strains and plasmids used in the study

Strain or plasmid	Characteristics	References
Streptococcus suis		
SC84	Serotype 2 strain isolated from a patient with streptococcal toxic shock-like syndrome in China	[47]
1135776	Serotype 9 strain isolated from pig following sudden death in Canada	This study
S2Δ*agI/II*	SC84-derived strain carrying an in-frame deletion of the *agI/II* gene	This study

Strain or plasmid	**Characteristics**	**References**
S9Δ*agI/II*	1135776-derived strain carrying an in-frame deletion of the *agI/II* gene	This study
S2CΔ*agI/II*	SC84-derived strain carrying pOri23-S2*agI/II*	This study
S9CΔ*agI/II*	1135776-derived strain carrying pOri23-S9*agI/II*	This study
Escherichia coli		
TOP10	Host for pCR2.1 and pSET4s derivatives	Invitrogen
MC1061	Host for pOri23 derivatives	[16]
BL21(DE3)	Host for pET151 derivatives	Invitrogen
Plasmids		
pET151	Ap^r, pBR322 *ori*, T7 promotor	Invitrogen
pCR2.1	Ap^r, Km^r, pUC *ori*, *lacZ*ΔM15	Invitrogen
pSET4s	Spc^r, pUC *ori*, thermosensitive pG+host3 *ori*, *lacZ*ΔM15	[37]
pOri23	Erm^r, ColE1 *ori*, *P23*	[28]
pET151–S2*agI/II*	pET151 carrying the S2 *agI/II* gene	This study
pSET4s–S2*agI/II*	pSET4s carrying regions upstream and downstream of the S2 *agI/II* gene	This study
pSET4s–S9*agI/II*	pSET4s carrying regions upstream and downstream of the S9 *agI/II* gene	This study
$pOri23_{spc}$–S2*agI/II*	pOri23 carrying the S2 *agI/II* gene as well as its promotor and terminator	This study

Strain or plasmid	Characteristics	References
pOri23$_{spc}$–S9*agI/II*	pOri23 carrying the S9 *agI/II* gene as well as its promotor and terminator	This study

Bioinformatics analyses

In silico analyses of AgI/II-coding DNA sequences (CDS) in *S. suis* genomes were performed using BLASTN (expected threshold $< 10^{-3}$) as previously described [17]. The *S. suis* nucleotide collection nr/nt database available in GenBank (taxid 1307) was queried for *S. suis* genomes. Alongside, a bank of *S. suis* serotype 2 North American ST25 and ST28 strains isolated from diseased pigs whose genomes were previously published [18, 19] were also queried. Moreover, BLASTN was used to detect homologies with genes coding for AgI/II or orthologues that have already been described in other bacterial species: *S. mutans* SpaP (accession number NC_004350.2), *S. gordonii* SspA and SspB (accession number CP000725.1), *S. pyogenes* (accession number NC_007296.1), *S. agalactiae* (accession number AAJP01000002.1), and *Enterococcus faecalis* (accession number AY855841.2). Examination of CDS carriage by putative integrative and conjugative elements (ICEs) was conducted using the ICEberg database [20], followed by BLASTN using the *S. suis* serotype 2 SC84 (accession number GCA_000026725.1) and serotype 9 D12 (accession number GCA_000231905.1) genomes as queries. Protein domains were analyzed using the NCBI conserved domain database with the help of the BatchCD tool [21]. Cell wall anchored domains were predicted using CW-PRED [22], while transmembrane domains and signal peptide cleavage sites were detected using the TMHMM [23] and the SignalP [24] tools, respectively. The Expasy bioinformatics resource portal was used to determine the theoretical protein molecular weight [25].

DNA manipulations

Chromosomal *S. suis* DNA was prepared using standard methods [26] or InstaGene matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Plasmid

DNA preparations and purification of PCR amplicons were performed using the QIAprep Spin Miniprep Kit and the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions. Oligonucleotide primers (listed in Additional file 2) were purchased from Integrated DNA Technologies (Coralville, IA, USA). Primers were designed from the available *S. suis* serotype 2 (strain SC84) and serotype 9 (strain D12) genomes. DNA ligations and transformation of competent *E. coli* were performed as previously described [27]. Sequencing reactions were carried out using an ABI 3730xl Automated DNA Sequencer and the ABI PRISM Dye Terminator Cycle Version 3.1 (Applied Biosystems, Foster City, CA, USA) and analyses of sequences performed using the BioEdit© software and/or BLASTN.

Supplemental Table S2. List of primers used in this study. Restriction sites are underlined and in bold.

Name	Sequence	Use
Scr_S9*agI//II* F	AGAGACAATTGCACGAGGCT	Screening of *agI//II* gene in serotype 9 strains
Scr_S9*agI//II* R	GTGGGTCCTCAGGCATGAAA	Screening of *agI//II* gene in serotype 9 strains
S2Δ*agI//II*_1	GAGTTGCTGCTTCACAGTA	Construction of S2Δ*agI//II* mutant
S2Δ*agI//II*_2	ACTCTTACGAAAGACGTGTT	Construction of S2Δ*agI//II* and S9Δ*agI//II* mutants
S2Δ*agI//II*_3	TGCCAAATACAGGGGAACA	Construction of S2Δ*agI//II* and S9Δ*agI//II* mutants
S2Δ*agI//II*_4	AGGACTAGCCTGGACAAA	Construction of S2Δ*agI//II* and S9Δ*agI//II* mutants
S2Δ*agI//II*_5	TTTTT**AAGCTT**TATCTTCTGAG AGTGTTATTTGAT	Construction of S2Δ*agI//II* and S9Δ*agI//II* mutants
S2Δ*agI//II*_6	TAGAATACCAGCTCCAATAAAG TGTCCTTCTTTTCTTTTTTC	Construction of S2Δ*agI//II* mutant
S2Δ*agI//II*_7	AGAAAAGAAGGACACTTTATT GGAGCTGGTATTCTACTTGGTA	Construction of S2Δ*agI//II* mutant
S2Δ*agI//II*_8	TTTTT**AAGCTT**CTGAACAGATC TTTTGATTCCCT	Construction of S2Δ*agI//II* mutant
S9Δ*agI//II*_1	ATACCTTCTTCGGAATCTGCT	Construction of S9Δ*agI//II* mutant
S9Δ*agI//II*_6	AAGTAGAATACCAGCTCCAATA AATAGTCCTTCTTTTCTTTTTTA TGTAGC	Construction of S9Δ*agI//II* mutant
S9Δ*agI//II*_7	AAAAAGAAAAGAAGGACTATT TATTGGAGCTGGTATTCTACTT GGTA	Construction of S9Δ*agI//II* mutant
S9Δ*agI//II*_8	TTTTT**AAGCTT**GTGAAGTACCT GAACAGGT	Construction of S9Δ*agI//II* mutant
pET151_S2*agI//II*_Δ*CWA*_F	CACCGCTGTTGTTGCTTGGGGT	Cloning S2 *agI//II* gene in pET151
pET151_S2*agI//II*_Δ*LPXTG*_R	CTACTACTAGGCTGAAATTGAC ACGAGGCCATT	Cloning S2 *agI//II* gene in pET151
pOri23_S2*agI//II*_EcoRI_F	GGCGC**GAATTC**TCGACGCGGT ATAGAATTCCT	Cloning S2 *agI//II* gene in pOri32
pOri23_S2*agI//II*_PstI_R	GGCGC**CTGCAG**TCAAACCATC TTGGTCTGA	Cloning S2 *agI//II* gene in pOri32
pOri23_S9*agI//II*_EcoRI_F	GGCGC**GAATTC**TCAACTCGGA AGATAATGCCT	Cloning S9 *agI//II* gene in pOri32
pOri23_S9*agI//II*_PstI_R	GGCGC**CTGCAG**TCAAACCATC TTGGTCTGA	Cloning S9 *agI//II* gene in pOri32
Spc^R_EcoRI_F	GGCGC**GAATTC**GTTCGTGAAT ACATGTTATA	Amplification of spectinomycin^R gene from pSET4s

Generation of the isogenic and complemented *agI/II*-deficient mutant strains

For precise in-frame deletions of the *agI/II* genes in the *S. suis* serotype 2 strain SC84 and serotype 9 strain 1135776, regions upstream and downstream of the genes were amplified and fused by overlap-extension PCR. The amplification products were subcloned into vector pCR2.1 (Invitrogen), excised using *Hin*dIII (Promega, Madison, WI, USA), and cloned into the thermosensitive gene replacement vector pSET4s as previously described [27]. The resulting serotype 2 and serotype 9 pSET4S-*agI/II* vectors were introduced into recipient serotype 2 and 9 strains, respectively. Allelic replacement and absence of AgI/II expression in resulting serotype 2 and serotype 9 *agI/II*-deficient mutants were confirmed by sequencing and Western blot, respectively.

The pOri23 plasmid [28], which carries a gene conferring resistance to erythromycin, was used for complementation assays. A DNA fragment composed of the full sequence of the *agI/II* genes, as well as their putative endogenous promotors and terminators was cloned into pOri23 using the *Eco*RI and *Pst*I restriction enzymes (two constructs, one for the serotype 2 *agI/II* and another for the serotype 9 *agI/II*). Since the serotype 9 strain used is highly resistant to erythromycin (data not shown), and several reports have described increased resistance to this antimicrobial among serotype 2 strains [29, 30], a spectinomycin resistance cassette derived from pSET4s was introduced into the pOri23–S2*agI/II* and pOri23–S9*agI/II* plasmids. Following subcloning steps using *E. coli* MC1061, the generated $pOri23_{spc}$–S2*agI/II* and $pOri23_{spc}$–S9*agI/II* plasmids were then introduced into the S2Δ*agI/II* and S9Δ*agI/II* strains to generate the complemented S2CΔ*agI/II* and S9CΔ*agI/II* strains, respectively.

Cloning, expression, and purification of the His-tagged recombinant AgI/II protein and production of polyclonal mono-specific antibodies

A 4430 bp fragment of the serotype 2 *agI/II* gene, excluding the sequences coding for the cell wall anchorage and the LPXTG domains, was cloned into the pET151 expression vector (Invitrogen) according to the manufacturer's instructions (Figure 1). Protein synthesis was induced using 0.5 mM of isopropyl β-D-1-thiogalactopyranoside and cells lysed using

lysozyme (Sigma-Aldrich) and sonication. The resulting recombinant His-tagged AgI/II, henceforth rAgI/II, was purified by affinity chromatography using the His-Bind Resin Chromatography Kit (Novagen, Madison, WI, USA,) according to manufacturer's instructions. Protein purity was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis following dialysis. Protein concentration was determined using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Rabbits were inoculated with the purified rAgI/II to produce a mono-specific polyclonal serum as previously described [31]. This serum was then used to verify presence of the protein in wild-type, isogenic *agI/II*-deficient mutants, and complemented strains by Western blot as previously described [32].

Cell surface hydrophobicity

The relative surface hydrophobicity of the *S. suis* wild-type strains and *agI/II*-deficient mutants was determined by measuring their adsorption to *n*-hexadecane as previously described [33]. A serotype 2 non-encapsulated mutant strain showing a high percentage of hydrophobicity was used as a positive control [33].

In vitro pathogenesis assays

Self-aggregation and biofilm assays

For the self-aggregation assays, overnight cultures of *S. suis* were washed twice with phosphate-buffered saline (PBS), pH 7.3, and re-suspended in THB to obtain an optical density (OD) at 600 nm of 0.05. Samples were incubated at 37 °C for 24 h under static conditions and self-aggregation quantified as previously described [34]. Biofilm formation capacity was determined as previously described [35] in the absence or presence of 2 mg/mL of porcine fibrinogen (Sigma-Aldrich).

***S. suis* aggregation to soluble porcine salivary agglutinins**

Saliva was obtained from pigs as previously described [36] with a few modifications. Briefly, cotton ropes were suspended for 30 min to allow a total of 80 growing pigs from a high health status herd with no recent history of endemic *S. suis* disease to chew. No clinical

signs of disease were present during collection. Whole saliva was decanted and impurities eliminated by centrifugation at 8000×*g* for 20 min at 4 °C. pSAGs were then purified from clarified saliva as previously described for human salivary agglutinins using *S. mutans* [37]. The pSAGs were dialyzed in PBS and the concentration determined using the Pierce BCA Protein Assay Kit. Bacterial aggregation was quantified every 20 min for 1 h in the absence or presence of pSAGs [37].

Evaluation of *S. suis* adhesion to extracellular matrix proteins, porcine salivary agglutinins, and gp340-derived SRCRP2 peptide by ELISA

Bacterial cultures were produced as previously described [38]. Formaldehyde-killed bacteria were washed using either PBS-T (PBS containing 0.05% Tween-20) for experiments involving extracellular matrix proteins (ECM), or TBS-T (10 mM Tris–HCl, 150 mM NaCl, pH 7.5 containing 0.1% Tween-20) supplemented with 1 mM $CaCl_2$, for experiments involving pSAGs and the gp340-derived SRCRP2 peptide [39]. Maxisorp flat-bottom microtiter plates (NUNC, Rochester, NY, USA) were coated with 12.5 µg/mL of human plasma fibronectin (Sigma-Aldrich), 15 µg/mL of human type I collagen (Corning, Corning, NY, USA), 1 mg/mL of porcine fibrinogen or 50 µg/mL of pSAGs, all diluted in carbonate coating buffer (0.1 M, pH 9.6), or with 200 µg/mL of the SRCRP2 peptide (Bio Basic Canada Inc., Markham, ON, Canada) diluted in water, overnight at 4 °C. After washing with PBS-T or TBS-T and blocking with non-fat dry milk, bacterial suspensions equivalent to 1×10^8 CFU/mL were added to the plates and incubated at 37 °C for 2 h. Subsequent steps were undertaken as previously described [38] using serotype 2 or 9 specific rabbit antisera and the OD at 450 nm determined.

Acid stress killing assay

The ability of *S. suis* to withstand acid challenge was determined as previously described with some modifications [39]. Briefly, *S. suis* strains were grown in THB, washed twice with PBS, and adjusted to a concentration of 1×10^8 CFU/mL. Cells were then resuspended in 0.1 M glycine buffer adjusted to either pH 3.0 or 5.0 and incubated at 37 °C.

Surviving bacteria were accurately determined using an Autoplate 4000 Spiral Plater (Spiral Biotech, Norwood, MA, USA).

Cell adhesion and invasion assays

The newborn porcine tracheal epithelial cell line (NPTr) was cultured until confluent as previously described [40]. Cells were infected with *S. suis* as previously described with minor modifications [41]. Briefly, PBS-washed NPTr cells were incubated at 37 °C with 5% CO_2 and infected with *S. suis* at a multiplicity of infection of 10. After 2 h of incubation, wells were washed with PBS to remove non-associated bacteria. For adhesion assays, cells were lysed with 1 mL of cold water, while the invasion assay was performed using the antibiotic protection method as previously described [40], and associated or intracellular bacteria enumerated as described above.

Intranasal colonization in a porcine model of 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, which approved the protocols and procedures used herein (permit number RECH-1570). Four-week old pigs (providing from the same high health status herd mentioned above) were used. The 10 pigs were randomly separated into two rooms upon arrival and their nasal cavities, saliva, and tonsils swabbed to confirm absence of serotype 9. The *S. suis* serotype 9 wild-type strain 1135776 and *agI/II*-deficient mutant were cultured as previously described [42] to obtain a final concentration of 2×10^9 CFU/mL. Intranasal infections were carried out as previously described with some modifications [43]. Pigs were inoculated with 1 mL of 2% acetic acid per nostril 1 h prior to infection with 1 mL per nostril of either the wild-type or the S9Δ*agI/II* mutant strain.

Nasal cavities were swabbed using sterile cotton-tipped applicators. Swabs were placed in sterile tubes containing PBS supplemented with 0.1% bovine serum albumin and immediately cultured. Serial dilutions of swab samples (10^0–10^{-6}) were plated on Colombia

agar supplemented with 5% defibrinated sheep blood (Cedarlane, Burlington, ON, Canada), *Streptococcus* selective reagent SR0126 (Oxoid, Hampshire, UK), and selected antibiotics to which the serotype 9 strain is resistant at the concentrations used (50 µg/mL spectinomycin, 5 µg/mL erythromycin, 0.2 µg/mL penicillin G, and 1 µg/mL tetracycline). After incubation for 24 h at 37 °C with 5% CO_2, plates containing 30–300 colonies were selected. Suspected alpha-hemolytic colonies were enumerated and 10 *S. suis*-like colonies per plate were sub-cultured and tested by coagglutination assay using anti-*S. suis* serotype 9 rabbit serum as previously described [44]. Three weeks post-infection, pigs were euthanized and tonsils recovered. Tonsil samples were processed as previously described [45] and *S. suis* serotype 9 carriage evaluated as described above.

Statistical analyses

At least three independent biological replicates were performed for each experiment and results expressed as mean ± standard error of the mean (SEM). Raw data were analyzed using the non-parametric statistical Mann–Whitney test. Statistical differences are defined as being greater than $p < 0.05$.

Declarations

Ethics approval

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, which approved the protocols and procedures used herein (permit number RECH-1570). A total of 10 pigs were used in this study.

Funding

This study was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) to MG (grant #154280).

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ANNEXE; ARTICLE XII

The bias of experimental design, including strain background, in the determination of critical *Streptococcus suis* serotype 2 virulence factors

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Publié dans PLoS ONE. **2017**. 12(7): e0181920. doi.org/10.1371/journal.pone.0181920

Détails sur le rôle du candidat dans la conception de l'article :
Je suis coauteur de l'article. J'ai contribué à la construction et à la caractérisation des mutants.

Abstract

Streptococcus suis serotype 2 is an important porcine bacterial pathogen and emerging zoonotic agent mainly responsible for sudden death, septic shock, and meningitis. However, serotype 2 strains are genotypically and phenotypically heterogeneous. Though a multitude of virulence factors have been described for *S. suis* serotype 2, the lack of a clear definition regarding which ones are truly "critical" has created inconsistencies that have only recently been highlighted. Herein, the involvement of two factors previously described as being critical for *S. suis* serotype 2 virulence, whether the dipeptidyl peptidase IV and autolysin, were evaluated with regards to different ascribed functions using prototype strains belonging to important sequence types. Results demonstrate a lack of reproducibility with previously published data. In fact, the role of the dipeptidyl peptidase IV and autolysin as critical virulence factors could not be confirmed. Though certain *in vitro* functions may be ascribed to these factors, their roles are not unique for *S. suis*, probably due to compensation by other factors. As such, variations and discrepancies in experimental design, including *in vitro* assays, cell lines, and animal models, are an important source of differences between results. Moreover, the use of different sequence types in this study demonstrates that the role attributed to a virulence factor may vary according to the *S. suis* serotype 2 strain background. Consequently, it is necessary to establish standard experimental designs according to the experiment and purpose in order to facilitate comparison between laboratories. Alongside, studies should include strains of diverse origins in order to prevent erroneous and biased conclusions that could affect future studies.

Introduction

Streptococcus suis is an important porcine bacterial pathogen and emerging zoonotic agent mainly responsible for sudden death (pigs), septic shock (humans), and meningitis (both species) [1]. Of the different described serotypes based on the presence of the capsular polysaccharide or its respective genes, serotype 2 is regarded as not only the most widespread worldwide, but also the most virulent, responsible for the majority of porcine and human cases of *S. suis* infection [2]. Using multilocus sequence typing, the distribution of the most important sequence types (STs) of *S. suis* serotype 2 has been determined worldwide [2]. Moreover, recent studies have evaluated the virulence of these important STs using well-characterized mouse models of infection, where virulence is defined based on the capacity of a strain to induce clinical disease and mortality [3, 4]. The ST7 strain responsible for the human outbreaks of 1998 and 2005 in China [5] is highly virulent whereas European ST1 strains are virulent; on the other hand, ST25 strains, typically recovered in North America, are of intermediate virulence [3].

Over the years, a multitude of virulence factors, presently totaling more than 150, have been described to be implicated in the *S. suis* serotype 2 pathogenesis in pigs and humans [6-9]. However, the lack of a clear definition regarding what constitutes a virulence factor for *S. suis*, which generally differs from one laboratory to another, and the fact that many of these have redundant roles, have greatly hindered the identification of truly “critical” virulence factors and created inconsistencies throughout the literature [9]. Indeed, of the different factors described so far, at least 76 have been reported to be implicated in virulence, while 35 of these were critical for virulence since their absence resulted in avirulence [9]. Alongside, many putative virulence factors are present in certain virulent strains but not in others, such as the suilysin, muramidase-released protein (MRP), and extracellular protein factor, which currently serve as virulence markers for Eurasian *S. suis* serotype 2 strains only, since these are often absent in North American strains [2, 9, 10]. In fact, the North American strains that do possess the MRP are associated with lower virulence [4, 10]. Moreover, the important roles played by a putative virulence factor might depend on the genetic background of the selected strain. Finally, the use of differing experimental designs, including *in vitro* assays, cell lines, and

animal models, have made it extremely difficult to accurately compare results between laboratories [9].

Herein, the involvement of two *S. suis* serotype 2 virulence factors previously described as being critical were evaluated with regards to different ascribed functions using prototype strains belonging to three of the most important STs (ST1, ST7, and ST25). These proteins, which served as tools, were chosen among the more than 150 putative virulence factors described for *S. suis* on the basis of being present in strains from these three backgrounds. The dipeptidyl peptidase IV (DPPIV), originally studied using a ST7 isolate recovered from a human case during the 2005 Chinese outbreak [11], is a serine protease widely distributed in eukaryotes and bacteria that has been suggested to contribute to bacterial pathogenesis [12]. Meanwhile, the autolysin [13], originally studied using a ST378 strain recovered from a diseased pig in China [14], is a peptidoglycan hydrolase implicated in various bacterial functions such as cell wall turnover, cell division, and cell separation [13]. Consequently, the aim of this study was to determine the bias of experimental design, including strain background, in the determination of *S. suis* serotype 2 virulence factors in order to better clarify the recently highlighted controversy caused by inconsistencies plaguing this field of research.

Results

The *S. suis* serotype 2 dipeptidyl peptidase IV and autolysin are not major fibronectin-binding adhesins, regardless of the sequence type of the strain used

Adhesion to host extracellular matrix (ECM) components is an important and often crucial initial step of the bacterial pathogenesis [8]. Amongst the different components of the ECM is plasma fibronectin, to which both the DPPIV of a ST7 strain and the autolysin of a ST378 strain were previously reported to bind [11, 13]. Results showed similar levels of adhesion to fibronectin between the wild-type ST1, ST7, and ST25 strains, as measured by ELISA (Fig 1). The role of the DPPIV and autolysin in binding human fibronectin was then evaluated using their respective isogenic mutants. In the presence of 10 µg/mL of fibronectin (concentration shown to be optimal for *S. suis*; data not shown), no significant differences were observed between the adhesion of the Δ*dppIV* or Δ*alt* mutants and their respective wild-type strains, regardless of the ST of the strain used (Fig 1). Similar results were obtained using lower concentrations of fibronectin (data not shown). These results suggest that the DPPIV and autolysin are not major human fibronectin-binding adhesins for *S. suis* serotype 2.

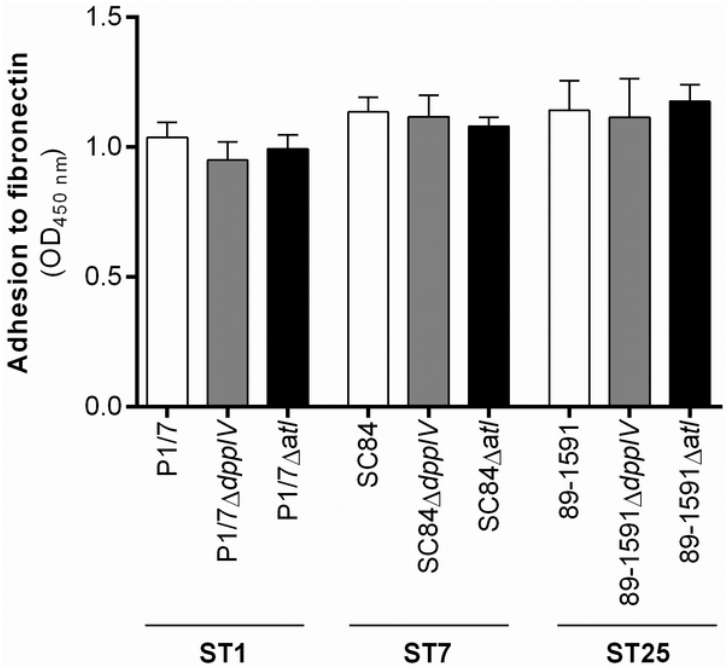

Fig 1. The *S. suis* serotype 2 dipeptidyl peptidase IV and autolysin are not involved in adhesion to fibronectin, regardless of the sequence type (ST) of the strain used. Adhesion of different wild-type

strains and dipeptidyl peptidase IV (DPPIV)- or autolysin (Atl)-deficient mutants to human plasma fibronectin (10 μg/mL), as determined by ELISA after 2 h of incubation. The optical density (OD) was measured at 450 nm and values corrected using the appropriate controls. Results are expressed as mean ± SEM obtained from three independent experiments.

The *S. suis* serotype 2 dipeptidyl peptidase IV, unlike the autolysin, does not play a major role in adhesion to porcine tracheal epithelial cells

Adhesion to host cells is a requirement for subsequent interactions, including cell activation and establishment of the disease [8]. Indeed, it was previously suggested that both the DPPIV [11] and autolysin [13] are implicated in adhesion of *S. suis* serotype 2 to the human laryngeal epithelial cell line HEp-2. Herein, adhesion of the different wild-type strains and the role of the DPPIV and autolysin in adhesion to the porcine tracheal epithelial cell line NPTr was determined after 2 h of incubation with a MOI=10 and results expressed as percentage of adhered inoculum. Adhesion of the wild-type ST1 strain was greatest, with that of the ST7 strain being intermediate, while the ST25 strain adhered the least, adhesion of which was significantly lower than that of the wild-type ST1 strain only ($p < 0.01$) (Fig 2). Moreover, results showed that the autolysin, but not the DPPIV, plays an important role in the adhesion to NPTr for the three wild-type strains ($p < 0.001$) (Fig 2). In fact, adhesion of the autolysin-deficient mutants was only 0.6%, 0.6%, and 0.4% of the inoculum for the ST1, ST7, and ST25 strains, respectively, in comparison to 2.3%, 1.9%, and 1.4% for their respective wild-type strains. As such, adherence of the three mutant strains was reduced by more than 70%. The DPPIV mutants, however, showed no differences compared to their respective wild-type strains regarding adhesion to porcine epithelial cells.

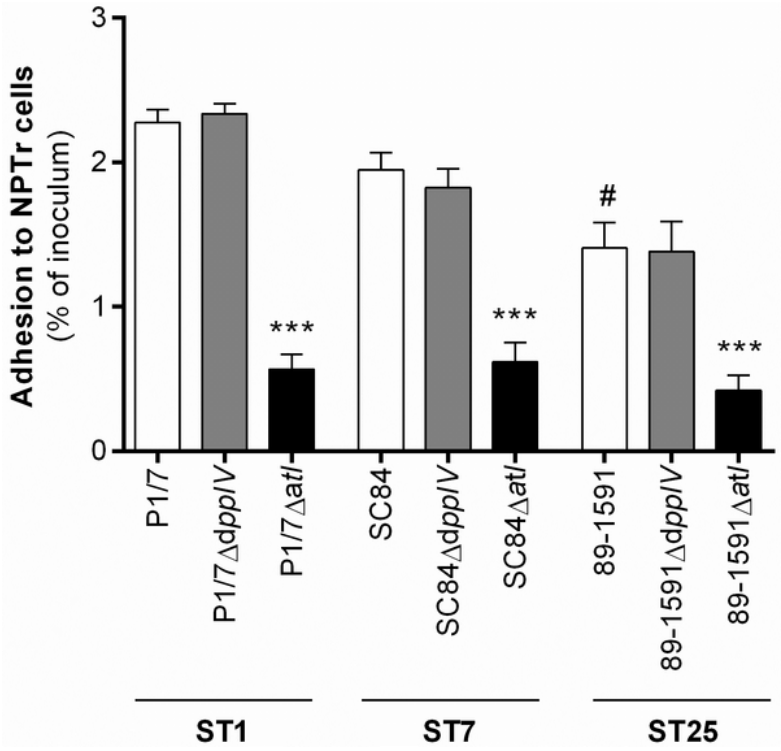

Fig 2. The *S. suis* serotype 2 dipeptidyl peptidase IV is not involved in adhesion to porcine tracheal epithelial cells, regardless of the sequence type (ST) of the strain used, unlike the autolysin. Adhesion of different wild-type strains and dipeptidyl peptidase IV (DPPIV)- or autolysin (Atl)-deficient mutants to porcine epithelial cells was evaluated after 2 h of incubation with bacteria (MOI=10). Results are expressed as mean ± SEM obtained from three independent experiments and represent the percentage of adhered inoculum. # indicates a significant difference ($p < 0.01$) between the wild-type ST1 strain P1/7 and ST25 strain 89-1591; *** ($p < 0.001$) between the wild-type strain and its Atl-deficient mutant.

The dipeptidyl peptidase IV is not involved in *S. suis* serotype 2 biofilm formation, while the implication of the autolysin is strain-dependent

Alongside adhesion to host ECM and cells, the capacity to form biofilm has been described as important for the *S. suis* pathogenesis, being involved in survival and propagation [8]. In order to enhance the biofilm formation capacity of the wild-type and mutant strains, culture medium was supplemented with porcine fibrinogen as previously described [26]. Results demonstrated that the wild-type ST1 strain produced significantly more biofilm than both the wild-type ST7 ($p < 0.05$) and ST25 strains ($p < 0.01$), though the ST7 strain produced more than the ST25 strain ($p < 0.01$) (Fig 3). While no data were available regarding a role of

the DPPIV in biofilm formation by *S. suis*, the autolysin was previously reported to be implicated using a *S. suis* serotype 2 ST378 strain [13]. Using the same experimental design as previously used for the autolysin, the involvement of these two putative virulence factors in the capacity of the three wild-type *S suis* strains to form biofilm was evaluated. While the DPPIV was not involved in biofilm formation, regardless of the sequence type of the strain used, the autolysin of the ST7 and ST25 strains participated in biofilm formation ($p < 0.001$), but not that of the ST1 strain (Fig 3).

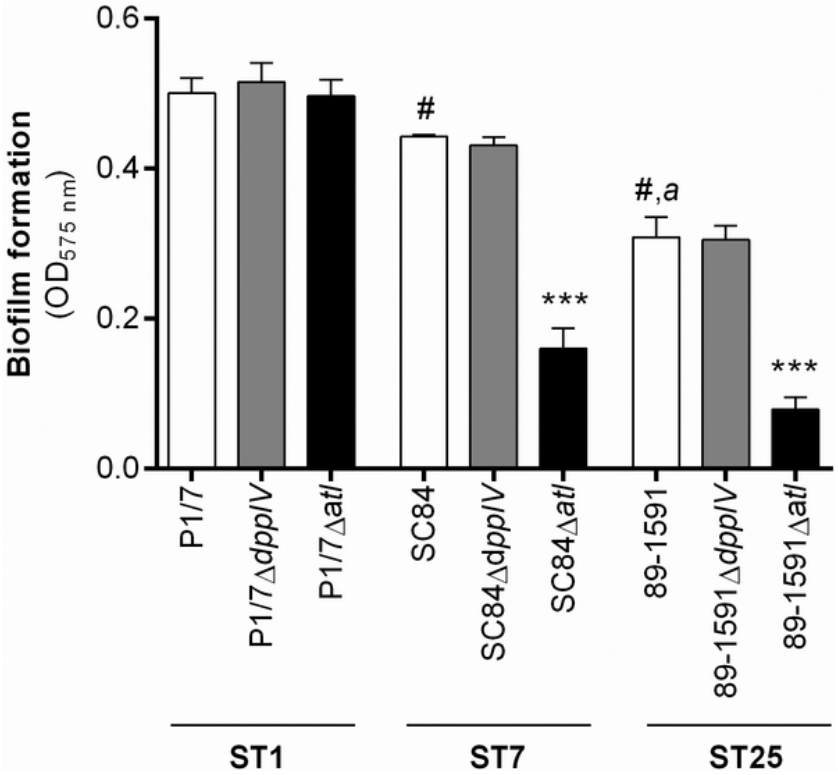

Fig 3. The dipeptidyl peptidase IV is not involved in *S. suis* serotype 2 biofilm formation, while implication of the autolysin is dependent on the sequence type (ST) of the strain used. Biofilm formation of different wild-type strains and dipeptidyl peptidase IV (DPPIV)- or autolysin (Atl)-deficient mutants in the presence of 2 mg/mL of porcine fibrinogen was evaluated after 24 h of incubation. The optical density (OD) was measured at 575 nm and values corrected using the appropriate controls. Results are expressed as mean ± SEM obtained from three independent experiments. # indicates a significant difference ($p < 0.05$) between the wild-type ST1 strain P1/7 and ST7 strain SC84 or ST25 strain 89-1591; *a* ($p < 0.01$) between the wild-type ST7 strain SC84 and ST25 strain 89-1591; *** ($p < 0.001$) between the wild-type strain and its Atl-deficient mutant.

The *S. suis* serotype 2 dipeptidyl peptidase IV and autolysin do not behave as critical virulence factors in an experimental model of *S. suis* serotype 2 infection

In order to evaluate the role of the DPPIV and autolysin in virulence, a well-characterized C57BL/6 mouse model of infection was used [4, 17]. In this model, mice succumb to septic shock during the systemic infection, after which surviving mouse are susceptible of developing meningitis. While no differences in survival were observed between mice infected with the wild-type ST1 (Fig 4A) and ST7 (Fig 4B) strains, results showed that the ST7 strain induced host death more rapidly than the ST1 strain. Moreover, the wild-type ST1 and ST7 strains caused significantly more mortality than the wild-type ST25 strain (Fig 4C) ($p < 0.05$): mortality caused by the ST25 strain was delayed and, unlike with the two other wild-type strains, partially due to the development of meningitis. For the virulent European ST1 strain (Fig 4A) and the highly virulent Chinese ST7 strain (Fig 4B), no significant role of the DPPIV and autolysin as critical virulence factors was observed. Moreover, this was also the case for the DPPIV of the intermediate virulent North American ST25 strain (Fig 4C). Meanwhile, and surprisingly, the ST25 autolysin-deficient mutant caused significantly higher mortality ($p < 0.05$) than its wild-type strain (Fig 4C). These results were confirmed in a subsequent infection (data not shown). Given these results, the *S. suis* serotype 2 DPPIV and autolysin are not significantly involved in virulence using a C57BL/6 mouse model of infection.

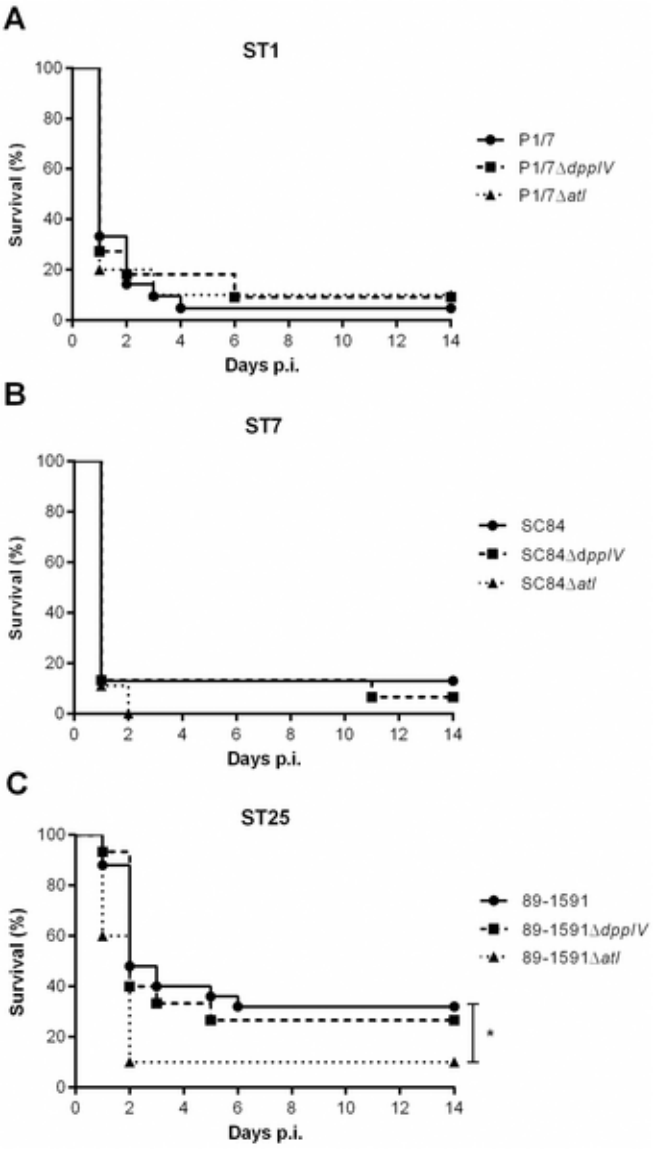

Fig 4. The *S. suis* serotype 2 dipeptidyl peptidase IV and autolysin are not implicated in host virulence in a C57BL/6 mouse model of infection, regardless of the sequence type (ST) of the strain, with the exception of a minor role for the autolysin of the ST25 strain. Survival of C57BL/6 mice infected with $5x10^7$ CFU of different wild-type and dipeptidyl peptidase IV (DPPIV)- or autolysin (Atl)-deficient mutants by intraperitoneal inoculation. (A) P1/7 (ST1) and its mutants, (B) SC84 (ST7) and its mutants, and (C) 89-1591 (ST25) and its mutants. * indicates a significant difference ($p < 0.05$) between the wild-type ST25 strain 89-1591 and its Atl-deficient mutant.

The *S. suis* serotype 2 ST25 autolysin hinders bacterial survival in blood

Given the higher virulence of the autolysin-deficient ST25 strain, blood bacterial burden, which when uncontrolled may be responsible for *S. suis*-induced host death [4, 17], was evaluated 24 h p.i. for the wild-type ST25 strain and its two isogenic mutants. Indeed, blood bacterial burden was significantly higher ($p < 0.01$) in mice infected with the ST25 autolysin-deficient mutant than in those infected with either the wild-type strain or the DPPIV-deficient mutant, between which burdens were similar (Fig 5). This suggests that the autolysin might somewhat hinder survival of the ST25 strain in blood.

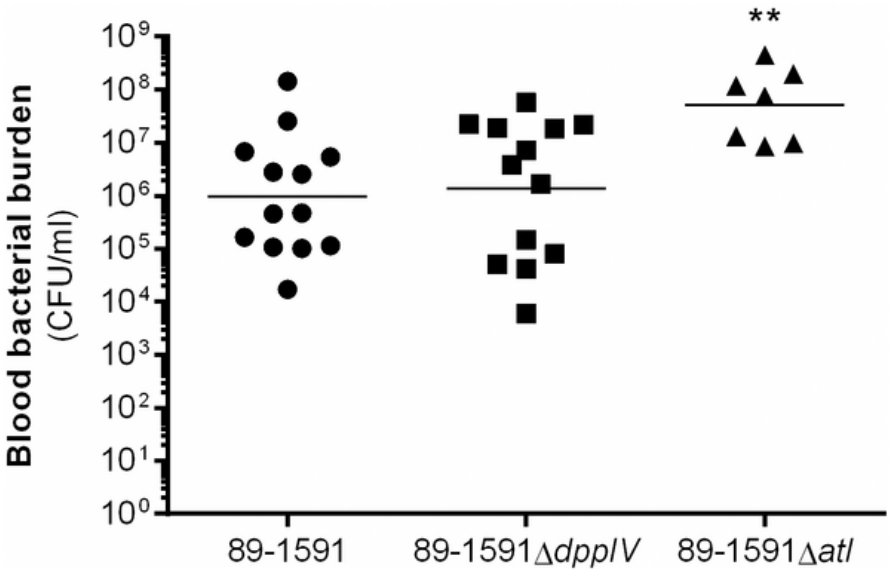

Fig 5. The autolysin of the ST25 strain, but not the dipeptidyl peptidase IV, hinders bacterial survival in the blood. Blood bacterial burden of surviving C57BL/6 mice 24 h following intraperitoneal inoculation of $5x10^7$ CFU of the ST25 strain 89-1591 and its dipeptidyl peptidase IV (DPPIV)- or autolysin (Atl)-deficient mutants. Results are expressed as geometric mean. ** indicates a significant difference ($p < 0.01$) between the wild-type ST25 strain 89-1591 and its Atl-deficient mutant.

Discussion

Though a multitude of virulence factors have been described for *S. suis* serotype 2, a clear definition of what constitutes a critical virulence factor is still lacking for this pathogen. This confusion has led to inconsistencies throughout the literature, resulting in a controversy that has only just been highlighted [9]. Consequently, the involvement of two *S. suis* serotype 2 factors, the DPPIV and autolysin, described as critical for virulence, was evaluated with regards to previously ascribed bacterial functions implicated in the *S. suis* pathogenesis using strains belonging to three important STs.

It is important to mention that the study by Ge *et al.* regarding the *S. suis* serotype 2 DPPIV was conducted using a ST7 strain (05ZYH33) isolated from the 2005 Chinese human outbreak [11]. Similarly, the ST7 strain used in the present study (SC84) was also isolated from a human case of streptococcal toxic shock-like syndrome during the same outbreak [19]. Since different isolates recovered from this outbreak have been reported to be highly similar [5], the influence of the background should be minimal between these two strains. Meanwhile, the strain originally used to study the *S. suis* serotype 2 autolysin by Ju *et al*. is a ST378 (HA9801) according to the *S. suis* Multilocus Sequence Typing Website (http://ssuis.mlst.net) [13]. This ST was never reported beforehand and has not been reported since. To facilitate comparison of the methodologies used between the previous [11, 13] and present studies, the main experimental designs of the different assays are listed in Table 1. Since mRNA expression of *dppIV* and *atl* is similar between the three wild-type strains under the growth conditions used in this study, the differences observed herein are probably due to other variations between the strains, such as the presence/absence of putative virulence factors and/or differential expression of these factors.

Table 1. Comparison of the experimental design used in the previous and present studies to evaluate the role of the dipeptidyl peptidase IV and autolysin as virulence factors for *S. suis* serotype 2

Experimental design	**Previous study – Dipeptidyl peptidase IV [11]**	**Previous study – Autolysin [13]**	**This study**
Sequence type (strain)	ST7 (05ZYH33)	ST378 (HA9801)	ST1 (P1/7) ST7 (SC84) ST25 (89-1591)
Adhesion to human fibronectin	Recombinant protein	Recombinant protein	Whole bacteria
	ELISA assay	Western blot	ELISA assay
Adhesion to epithelial cells	HEp-2 (Human laryngeal cells)	HEp-2 (Human laryngeal cells)	NPTr (Porcine tracheal cells)
	MOI=10	MOI=100	MOI=10
	1 h or 2 h of incubation?	3 h of incubation	2 h of incubation
	% adhered inoculum	% adhered inoculum? Adhesion of wild-type strain=100%?	% adhered inoculum
Biofilm formation	Not evaluated	Microtiter plate assay after 24 h of incubation	Microtiter plate assay after 24 h of incubation
Virulence	Mouse Unknown (SPF), possibly BALB/c?	Zebrafish	Mouse C57BL/6
	Subcutaneous or intravenous? $1x10^8$ CFU/mouse?	Intraperitoneal $2x10^3$ to $2x10^7$ CFU/fish	Intraperitoneal $5x10^7$ CFU/mouse

In order to evaluate the reproducibility of previously published results with strains from different backgrounds, the role of the *S. suis* serotype 2 DPPIV [11] and autolysin [13] in binding human plasma fibronectin was evaluated using a virulent European ST1 strain, the highly virulent clonal ST7 strain, and an intermediate virulent North American ST25 strain. The similar capacity of the three wild-type strains to bind human plasma fibronectin suggests this characteristic might be common to *S. suis* serotype 2, indicating a possibly universal role in the pathogenesis of this bacterium. Moreover, no significant implication of the *S. suis* serotype 2 DPPIV or autolysin in binding human fibronectin was observed when using

isogenic mutants, regardless of the ST and fibronectin concentration. While these results cannot exclude those previously obtained using the recombinant DPPIV and autolysin as evaluated by ELISA and Western blot, respectively, they suggest that while the recombinant proteins themselves might bind human fibronectin, their absence is not sufficient to affect binding to this ECM component by *S. suis*. This lack of role when using isogenic mutants could be the result of compensation by one or more of the 18 factors currently known to bind fibronectin other than the DPPIV and autolysin: the fibronectin/fibrinogen-binding protein [27], enolase [28], Ssa (fibronectin-binding protein) [29], MRP [30], sortase A-anchored protein [31], catabolite control protein A [32], type II histidine triad protein [33], fructose-bisphosphate aldolase, lactate dehydrogenase, oligopeptide-binding protein OppA precursor, elongation factor Tu [34], sbp2 (putative pilin subunit) [35], translation elongation factor G, phosphoglycerate mutase, phosphoglycerate kinase, pyruvate dehydrogenase E1 component alpha subunit, and chaperonin GroEL [36]. Indeed, 29 different *S. suis* serotype 2 virulence factors have been described so far as binding ECM components [8, 9], which supports bacterial redundancy [37]. This redundancy was recently demonstrated for another putative virulence factor of *S. suis* serotype 2: the deletion of a single factor H-binding protein, of which ten have been described and another six have been proposed, is not sufficient to inhibit bacterial binding to factor H [38-41]. In fact, the simultaneous deletion of two of these genes, alongside a triple knockout for the capsular polysaccharide (also reported to bind factor H), remained insufficient to abolish binding to factor H, suggesting compensation by at least another bacterial factor, most probably one or more of these described proteins [38]. Consequently, a descriptive role for a bacterial protein alone is probably not sufficient to claim an important role in the pathogenesis of the infection, especially when other factors with redundant functions have already been described.

Adhesion to ECM components may subsequently led to interactions with host cells, which is an important step of bacterial pathogenesis [8]. It was previously demonstrated that the DPPIV of the ST7 strain and the autolysin of a ST378 strain are both implicated in adhesion to the human laryngeal epithelial cell line HEp-2 [11, 13]. Interestingly, the percentage of adhesion to epithelial cells obtained herein varied between STs, indicating a role of strain background. In fact, the ST1 and ST7 strains, which are virulent and highly virulent,

respectively, adhered more than the intermediate virulent ST25 strain. Since adhesion to host cells may lead to cell invasion, differences in adhesion might influence host dissemination and virulence [6, 8]. Results obtained herein demonstrate that the DPPIV was not involved in adhesion to epithelial cells, regardless of the methodology used being similar (MOI and incubation time) and the ST being the same to that previously described [11]. However, certain differences in methodology still exist, such as the number of washes prior to cell lysis, the volume of water used to lyse the cells and, most notably, the origin of the cells used: human laryngeal epithelial cells versus porcine tracheal epithelial cells [11]. Though these two cell lines are both epithelial cells derived from the respiratory epithelium, it is impossible to ascertain that no other differences exist, such as histological differences between the trachea and larynx and the method used to immortalize the cells. Although it has been reported that adhesion to porcine and human epithelial cells by *S. suis* serotype 2 may be similar [15], the HEp-2 cells may not be an appropriate model for evaluating the role of all putative *S. suis* virulence factors since the respiratory route of infection has not been demonstrated for humans [9]. Consequently, these problems suggest that the experimental design used should be justified and the methodology standardized to ease comparison between studies and laboratories.

Meanwhile, a role of the autolysin in adhesion to host cells was confirmed when using porcine epithelial cells, and this for all three ST tested. Interestingly, it was previously reported that absence of the autolysin resulted in adhesion of only 50% of the inoculum by the mutant strain, while 100% of the wild-type strain inoculum adhered after 3 h of incubation [13]. These results greatly differ from those obtained in this study, in which approximately only 2% of the different wild-type strain inoculums and 0.5% of the autolysin-deficient mutant inoculums adhered to the epithelial cells. In fact, the high *S. suis* adhesion levels to epithelial cells reported by Ju *et al.* have never been observed by other researchers [13]. An hypothesis explaining the results of Ju *et al.* is that the elevated initial MOI (MOI=100) and longer incubation time (3 h) may have led to bacterial replication within the wells [13]. Interestingly, despite differences in methodology and the origin of the cells, results obtained in this study arrived to the same conclusions for all three STs tested, suggesting that the role of this putative virulence factor in adhesion to epithelial cells might be universal for *S. suis* serotype 2.

Alongside adhesion to host ECM and cells, the capacity to form biofilm is an essential step of the bacterial pathogenesis involved in survival and propagation of the pathogen [8]. Interestingly, the three wild-type strains produced varying levels of biofilm, indicating a role of strain background concerning this capacity. These differences imply that choice of strain can have an important effect on the results obtained when evaluating certain characteristics or functions of *S. suis*. Moreover, the DPPIV was determined not to be involved in biofilm formation, regardless of the ST of the strain used. Though no role in biofilm formation had been attributed to this *S. suis* protein in the past, the lack of evaluation could have suggested otherwise, as exemplified by *Porphyromonas gingivalis*, for which the DPPIV is clearly involved in biofilm formation [42]. On the other hand, the autolysin was implicated in biofilm formation for the ST7 and ST25 strains, but not the ST1 strain. While the autolysin was previously reported to be implicated in biofilm formation by an ST378 strain, its absence resulted in a 25% decrease of production for the latter [13], while a decrease corresponding to nearly 70% of the biofilm formed by the wild-type ST7 and ST25 strains was observed herein. These results indicate a strain-dependent role of the *S. suis* serotype 2 autolysin with regards to this bacterial function. Consequently, these results demonstrate the impact of strain background and the bias introduced by this choice when evaluating virulence factors. This is important given that most studies regarding the evaluation of *S. suis* serotype 2 virulence factors have used ST1 or ST7 strains only.

When evaluating the implication of bacterial virulence factors, the ultimate demonstration remains the use of *in vivo* infection models. However, there exists a vast variety of *S. suis* serotype 2 animal infection models, which has complicated comparison of results. Of these the mouse is one of the most popular, with the inbred C57BL/6 breed being commonly used [3, 4, 17, 43]. Firstly, results obtained herein confirm previous studies in which the ST1 and ST7 strains were reported to both be virulent, with the ST7 strain inducing mortality more rapidly than the ST1 strain [3, 4]. Moreover, the wild-type ST25 strain caused less mortality and in a delayed time due to an important number of cases of meningitis, as previously reported [3, 4]. Unlike previously reported for a ST7 strain, results obtained herein demonstrate that the DPPIV is not implicated in virulence and host death, regardless of the ST of the strain used [11]. It is worth mentioning that unlike the C57BL/6 mice used in this study,

Ge *et al*. only specify using specific pathogen free-mice [11]. It must be presumed that these mice are BALB/c since this is the breed used for the immunization experiments conducted within the same publication [11]. C57BL/6 mice, which were used in the present study, are reliable for *S. suis* studies as they exhibit a prototypical Th1 immune response and a strong pro-inflammatory response [17, 44, 45]. On the other hand, BALB/c mice are the prototypical Th2 mouse breed [44]. As such, the innate immune response differs between these two breeds: C57BL/6 mice produce higher levels of the pro-inflammatory cytokine tumor necrosis factor (TNF) and the Th1 cytokine interleukin (IL)-12p70, in comparison to BALB/c mice [44, 46]. Moreover, macrophages isolated from C57BL/6 mice produce effector molecules required for bacterial killing, including nitric oxide, whereas those from BALB/c do not, resulting in impaired bactericidal activity of the latter [44].

In addition, the route of infection may also differentially affect the conclusions. Herein, bacteria were inoculated via the intraperitoneal route (IP), while the route of inoculation used by Ge *et al*. although not clearly stated, was probably intravenous (IV) [11]. Though bacteria will reach the bloodstream following IP inoculation via lymphatic drainage, the initial cell types activated will differ: IP inoculation results in activation of peritoneal macrophages while IV injection leads to immediate stimulation of blood leukocytes [47]. Indeed, it was previously reported that the route of infection had an effect on disease development following Group B *Streptococcus* infection [48, 49]. To our knowledge, the IV route of inoculation for *S. suis* in mice has been used in only a limited number of studies [50-52], while most mouse studies have used the IP route of infection for *S. suis*, as reviewed by Segura *et al*. [9].

Meanwhile, the role of the autolysin of a ST378 strain in virulence was previously evaluated using the zebrafish model of infection [13], in which the autolysin-deficient mutant presented attenuated virulence. However, using the C57BL/6 mouse model of infection, results from the present study indicate that the autolysin does not critically contribute to virulence and does not participate in host death, independently of the ST of the strain tested. An important difference between these studies, alongside the ST of the strains used, is the experimental design and the use of animal model. Though zebrafish possess innate and adaptive immune responses [53], the genetic differences with pigs and humans are greater than

those between mice and pigs or humans [54]. Although ethical regulations facilitate the use of zebrafish over mice, the former are cold-blooded, are a model in which it is more difficult to conduct central nervous system studies (meningitis being the most important pathology caused by *S. suis* serotype 2), and are limited to lethal dose 50 studies [9]. Consequently, results obtained with zebrafish are difficult to extrapolate, which may limit their use in determining *S. suis* virulence factors.

Surprisingly, autolysin-deficiency resulted in increased virulence of the ST25 strain. This was unexpected since autolysin-deficient mutants of other pathogenic Gram-positive bacteria, including for LytA of *Streptococcus pneumoniae* [55] and AtlE of *Staphylococcus epidermidis* [56], were less virulent than their respective wild-type strains. Indeed, the *S. pneumoniae* autolysin, involved in cell wall remodeling, is responsible for the release of the pneumolysin, an important virulence factor of this pathogen [57]. Consequently, in the absence of the autolysin, it is possible that cell wall remodeling and protein secretion could be altered or halted, resulting in alteration of the ST25 bacterial strain surface architecture. It is well known that certain surface proteins of *S. suis* are important activators of the host cells [6, 8], and these may, by remaining attached to the bacteria or by being differentially expressed, contribute to inflammation and host death. Moreover, absence of the autolysin resulted in increased survival of the ST25 strain in blood, suggesting that this protein could play additional functions alongside those previously described for *S. suis* [13] as well as for other pathogenic streptococci [57]. Indeed, the *Staphylococcus saprophyticus* autolysin, Aas, was shown to bind sheep erythrocytes [58], although this function has not been described for *S. suis* so far. Further investigations will be required in order to better understand these differences in virulence and the possibly unique roles of this protein in ST25 strains.

Conclusions

This study reiterates the urgent need in arriving to a consensus regarding the definition of *S. suis* serotype 2 virulence factors. Inconsistencies abound in the literature due to differences obtained between laboratories, and these have created a controversy that has only just been highlighted. The main source of these differences are variations and discrepancies in experimental design, including *in vitro* assays, cell lines, and animal models, which greatly

affect the results, as demonstrated in this study for both the DPPIV and autolysin. Moreover, the use herein of different strain backgrounds has demonstrated that differences in bacterial characteristics and functions, alongside the role attributed to a virulence factor, may vary according to the *S. suis* serotype 2 strain. Consequently, it will be important to establish standard experimental designs, including methodology and appropriate cell lines and animal models, according to the experiment and purpose in order to facilitate comparison between laboratories. Alongside, studies should include strains of diverse origins in order to prevent erroneous and biased conclusions that could affect future studies. Finally, the use of alternative animal models cannot definitively exclude the role of a given *S. suis* virulence factor that may significantly contribute to disease during a natural infection in pigs. For example, the DPPIV has been reported to contribute to tissue degradation and perturbation of the host defense system [59], roles that although not critical in themselves, could significantly contribute to the final outcome of the natural infection by *S. suis*.

Materials and methods

Ethics statement

This study was 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 (Permit Number: Rech-1570).

Bacterial strains and growth conditions

The three well-characterized and highly encapsulated intermediate to highly virulent prototype wild-type *S. suis* serotype 2 strains and their isogenic mutants used in this study are listed in Table 2. Strains are minimally passaged and virulence of strains is routinely tested using cell-based assays and experimental infection models. The *S. suis* strains were cultured in Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON, Canada). For adhesion assays, bacterial cultures were prepared as previously described [15]. Briefly, upon reaching the mid-exponential phase, bacteria were washed twice with phosphate-buffered saline (PBS), pH 7.3, and resuspended in PBS for adhesion to fibronectin or cell culture medium (Gibco, Burlington, ON, Canada) for adhesion to porcine epithelial cells (described below). For experimental infections, early stationary phase bacteria were washed twice in PBS and resuspended in THB [4, 16, 17]. Bacterial cultures were appropriately diluted and plated on THB agar to accurately determine bacterial concentrations. mRNA expression of the *dppIV* and *atl* genes was determined to be similar between the three wild-type strains under the growth conditions used in this study as quantified by RT-qPCR (data not shown). The *Escherichia coli* strain and different plasmids used in this study are also listed in Table 2. When needed, antibiotics (Sigma, Oakville, ON, Canada) were added to the media at the following concentrations: for *S. suis*, spectinomycin at 100 μg/mL and chloramphenicol at 5 μg/mL; for *E. coli*, kanamycin and spectinomycin at 50 μg/mL and chloramphenicol at 30 μg/mL.

Table 2. Strains and plasmids used in this study.

Strains/plasmids	General characteristics	Reference
Streptococcus suis		
P1/7	Wild-type, virulent European ST1 strain isolated from a case of pig meningitis in the United Kingdom	[18]
P1/7Δ*dppIV*	Isogenic mutant derived from P1/7; in frame deletion of the *dppIV* gene	This study
P1/7Δ*atl*	Isogenic mutant derived from P1/7; in frame deletion of the *atl* gene	This study
SC84	Wild-type, highly virulent ST7 strain isolated from a case of human streptococcal toxic shock-like syndrome during the 2005 outbreak in China	[19]
SC84Δ*dppIV*	Isogenic mutant derived from SC84; in frame deletion of the *dppIV* gene	This study
SC84Δ*atl*	Isogenic mutant derived from SC84; in frame deletion of the *atl* gene	This study
89-1591	Wild-type, intermediate virulent North American ST25 strain isolated from a case of pig sepsis in Canada	[20]
89-1591Δ*dppIV*	Isogenic mutant derived from 89-1591; in frame deletion of the *dppIV* gene	This study
89-1591Δ*atl*	Isogenic mutant derived from 89-1591; in frame deletion of the *atl* gene	This study
Escherichia coli		
TOP 10	F^- mrcA Δ(mrr-hsdRMS-mcrBC) φ80 lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG	Invitrogen
Plasmids		
pCR2.1	Ap^r, Km^r, oriR(f1) MCS oriR (ColE1)	Invitrogen
pSET-4s	Thermosensitive vector for allelic replacement in *S. suis*. Replication functions of pG + host3, MCS oriR pUC19 lacZ Sp^R	[21]
p4Δ*dppIV*	pSET-4s carrying the construct for *dppIV* allelic replacement	This work
p4Δ*atl*	pSET-4s carrying the construct for *atl* allelic replacement	This work

DNA manipulations

S. suis genomic DNA was extracted using the InstaGene Matrix solution (BioRad Laboratories, Hercules, CA, USA). Mini-preparations of recombinant plasmids were carried out using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Restriction enzymes and DNA-modifying enzymes (Fisher Scientific, Ottawa, ON, Canada) were used according to the manufacturer's recommendations. Oligonucleotide primers (Table 3) were obtained from Integrated DNA Technologies (Coralville, IA, USA) and PCRs carried out with the iProof proofreading DNA polymerase (BioRad Laboratories, Mississauga, ON, Canada) or with the Taq DNA polymerase (Qiagen). Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using an ABI 310 Automated DNA Sequencer and the ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA).

Table 3. Oligonucleotide primers used for the construction of the *S. suis* dipeptidyl peptidase IV (*dppIV*) and autolysin (*atl*) mutants used in this study.

Name	**Primer Sequence (5'-3')**
ST1 & ST7Δ*dppIV*_1	GATCCAGCTCCAACTCCAATTC
ST1 & ST7Δ*dppIV*_2	TTGGGATCATGCACCACACC
ST1 & ST7Δ*dppIV*_3	CCCCCGGGGAAGTTCCGGCACCAATTCCAG
ST1 & ST7Δ*dppIV*_4	TCCGTCTACTTGCAAAATTCTCAATGGCAAATCCAC CTTG
ST1 & ST7Δ*dppIV*_5	TTGCCATTGAGAATTTTGCAAGTAGACGGAGGTC
ST1 & ST7Δ*dppIV*_6	CGGGATCCGTTCGGAACATACCAAAGGG
ST25Δ*dppIV*_1	CAATAAGAAGCCCAGCAAGAG
ST25Δ*dppIV*_2	GTTGCAAGTACCCTCATTTCC
ST25Δ*dppIV*_3	TCGCTTCCTTAAGCTGGTC
ST25Δ*dppIV*_4	TCCGTCTACTTGCAAAATTCTCAATGGCAAATC CACCTTG
ST25Δ*dppIV*_5	TTGCCATTGAGAATTTTGCAAGTAGACGGAGGTC
ST25Δ*dppIV*_6	GCCACTTGGTCAGACAAAG
ST1 & ST7Δ*atl*_1	CCAGTTGTAGCAGCAGAG
ST1 & ST7Δ*atl*_2	ACCAGCATGAAAAGAACAGATG
ST1 & ST7Δ*atl*_3	CATTTAACTGATGATGAAAAAG
ST1 & ST7Δ*atl*_4	ATACCAATTCATTACACCTTGCTCCTTTATGTATTTCACATGTAA
ST1 & ST7Δ*atl*_5	TTACATGTGAAATACATAAAGGAGCAAGGTGTAATGAATTGGTAT
ST1 & ST7Δ*atl*_6	GTACTTACAAAGAGCCAACAG
ST25Δ*atl*_1	GGAAGTGCTACACTACCGTC
ST25Δ*atl*_2	GACCAGCATGAAAAGAAC
ST25Δ*atl*_3	CGGAGCTGTTCCAGTT
ST25Δ*atl*_4	CAAGGCGAGTGTGGTACTCCTTTATGTATTTCACATGTAA
ST25Δ*atl*_5	TTACATGTGAAATACATAAAGGAGTACCACACTCGCCTTG
ST25Δ*atl*_6	GCAGATTTAATTACTTTCTTTAGC

Construction of the isogenic dipeptidyl peptidase IV and autolysin mutants

The DNA genome sequences of the wild-type *S. suis* strains were used. In-frame deletions of the *dppIV* or *atl* genes were constructed using splicing-by-overlap-extension PCRs as previously described [11, 13, 22]. Overlapping PCR products were cloned into pCR2.1 (Invitrogen, Burlington, ON, Canada), extracted with EcoRI, recloned into the thermosensitive *E. coli–S. suis* shuttle plasmid pSET4s, and digested with the same enzyme, giving rise to the knockout vector p4Δ*dppIV* or p4Δ*atl*. Electroporation of the three *S. suis* wild-type strains and procedures for isolation of the mutants were previously described [23]. Allelic replacement was confirmed by PCR and DNA sequencing analysis. Amplification products were purified with the QIAgen PCR Purification Kit (Qiagen) and sequenced as described above. mRNA expression of upstream and downstream genes flanking the *dppIV* and *atl* genes in the mutant strains was confirmed by RT-PCR, validating in-frame gene deletion (data not shown). Growth of the different mutant strains was similar to that of the wild-type strains (data not shown).

***S. suis* adhesion to human fibronectin (microtiter plate binding assay)**

Fibronectin adhesion assays were carried out as previously described [24]. Briefly, microtiter plates were coated with different concentrations of human plasma fibronectin (Sigma-Aldrich, St-Louis, MO, USA), ranging from 0 to 10 μg/mL, in 0.1 M carbonate buffer, pH 9.6. Formaldehyde-killed bacterial suspensions (equivalent to 1 x 10^8 colony forming units [CFU]/mL) of the different wild-type and mutant strains were added and the plates incubated for 2 h at 37°C. It was previously demonstrated that killing of *S. suis* using 0.2% formaldehyde does not affect its capacity to bind fibronectin [24]. An anti-*S. suis* serotype 2 rabbit serum followed by a horseradish peroxidase-labelled anti-rabbit IgG conjugate (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) were used. The anti-*S. suis* serum equally recognized the wild-type and mutant strains by enzyme-linked immunosorbent assay (ELISA) [24]. The enzyme substrate, 3,30,5,50-tetramethylbenzidine (Zymed, San Francisco, CA, USA) was used according to the manufacturer's instructions, the reaction stopped using 1 N H_2SO_4, and the optical density measured at 450 nm using a microtiter plate reader (Molecular Devices, Menlo Park, CA, USA). Uncoated wells served as background controls.

Casein-coated wells served as a control for non-specific adhesion of *S. suis* to protein-coated wells.

S. suis adhesion to porcine tracheal epithelial cells

The newborn porcine tracheal epithelial cell line (NPTr) was used and cultured until confluent as previously described [25]. Cells were infected with *S. suis* (10^6 CFU/well; multiplicity of infection [MOI]=10) by removing the cell culture medium, adding 1 mL of bacteria in cell culture medium without antibiotics, and incubating for 2 h at 37°C with 5% CO_2 as previously described [15]. Following incubation, cells were washed five times with PBS to remove non-adherent bacteria and lysed using 1 mL of sterile water. The lysates were appropriately diluted and plated on THB agar to quantify adhered bacteria. Alongside, the last wash was plated to confirm absence of non-adhered bacteria. The percentage of adhered bacteria was calculated according to the following: CFU recovered 2 h post-incubation / inoculum x 100% [11, 13].

S. suis biofilm formation capacity

The biofilm formation capacity of the different wild-type and mutant strains was determined as previously described [26]. Moreover, the protocol used was identical to that described by Ju *et al*., including the use of 2 mg/mL of porcine fibrinogen (Sigma-Aldrich), incubation for 24 h at 37°C, subsequent staining with crystal violet, and measurement of the optical density at 575 nm [13].

S. suis in vivo virulence mouse infections

A well-standardized C57BL/6 mouse model of infection was used [3, 4, 17]. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering through the use of humane endpoints, applied throughout this study when animals were seriously affected since mortality was not an endpoint measurement. No additional considerations or housing conditions were required. All staff members received the required animal handling training as administered by the University of Montreal Animal Welfare Committee. A total of 140 six-week-old male and

female C57BL/6 mice (Jackson Research Laboratories, Bar Harbour, MA, USA) were used (10 to 15 mice/group) in this study. Mice were inoculated with $5x10^7$ CFU via the intraperitoneal route and health and behavior monitored at least thrice daily until 72 h post-infection (p.i.) and twice thereafter until the end of the experiment (14 days p.i.) for the development of clinical signs of sepsis, such as depression, swollen eyes, rough hair coat, and lethargy. Mice were also monitored for the development of clinical signs of meningitis. Clinical scores were determined according to the grid approved by the University of Montreal Animal Welfare Committee (S1 Appendix) and required actions undertaken. Mice were immediately euthanized upon reaching endpoint criteria using CO_2 followed by cervical dislocation. No mice died before meeting endpoint criteria and all surviving mice were euthanized as described above at the end of the experiment (14 days p.i.). Blood samples were collected from the caudal vein of surviving mice 24 h p.i. and plated as previously described [4].

Statistical analyses

Significant differences were determined using the t-test, Mann-Whitney Rank sum test, one way ANOVA, and ANOVA on ranks, where appropriate. For *in vivo* virulence experiments, survival was analyzed using the LogRank test. A $p < 0.05$ was considered statistically significant.

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