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Identification and characterization of a new adhesin involved in the binding of *Streptococcus suis* to the extracellular matrix proteins

par

Miriam Esgleas Izquierdo

Département de pathologie et microbiologie

Faculté de médecine vétérinaire

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IDENTIFICATION AND CHARACTERIZATION OF A NEW ADHESIN INVOLVED IN THE BINDING OF *STREPTOCOCCUS SUIS* TO THE EXTRACELLULAR MATRIX PROTEINS

présentée par

MIRIAM ESGLEAS IZQUIERDO

a été évaluée par un jury composé des personnes suivantes :

Michaël Mourez, président-rapporteur

Marcelo Gottschalk, directeur de recherche

J. Daniel Dubreuil, codirecteur

Mariela A. Segura, membre du jury

Daniel Grenier, examinateur externe

John M. Fairbrother, représentant du doyen de la FÉSP

RÉSUMÉ

Streptococcus suis est un important pathogène porcin bactérien causant principalement des septicémies, des méningites, des endocardites et l'arthrite. Des 35 sérotypes connus, le sérotype 2 est le plus fréquemment isolé et associé à la maladie. *S. suis* est aussi reconnue comme étant un agent de zoonose. Les humains peuvent être infectés via par des petites coupures ou abrasions aux mains par ce pathogène lors de manipulations avec de la viande ou des carcasses de porc infectées. L'infection humaine peut être sévère et causer des méningites, des septicémies, des endocartides, la surdité (consequence de la méningite) et éventuellement la mort.

La pathogénèse de l'infection causée par *S. suis* n'est pas encore bien définie et plusieurs étapes sont probablement impliquées. Les mécanismes qui permettent à *S. suis* de se disséminer à partir des amygdales (le réservoir chez l'animal) ne sont pas bien compris. Il est admis que l'aptitude d'une bactérie à adhérer aux tissus de l'hôte est une étape critique de la plupart des infections microbiennes. Cette adhésion est considerée comme la première étape à une colonisation des muqueuses et peut aussi être la première étape à l'invasion des cellules de l'hôte, processus qui peut mener aux bactérémies ou aux septicémies. Un des types d'adhésines bactériennes les plus connues et les plus étudiés sont les protéines liant la fibronectine. De plus, la plupart des protéines liant la fibronectine décrites pour différents pathogènes ont été suggérées comme cibles potentielles de vaccins afin de prévenir l'infection bactérienne. En effet, il a été décrit que les anticorps liant ces protéines de surface ont la double activité de bloquer l'adhérence et d'augmenter la opsono-phagocytose.

À ce jour, trois types de protéines liant la fibronectine ont été identifiés chez les streptocoques: i) la famille MSCRAMM, renfermant un motif LPXTG médiant la liaison de cette próteine à la surface des bactéries par des sortases, ii) les protéines liant la choline et qui n'ont pas de motif LPXTG mais qui possèdent des répétitions liant la choline qui interagissent avec la phosphorylcholine des acides lipotéichoiques des bactéries à Gram positif et finalement, iii) un nouveau groupe de protéines nommé « anchorless » qui ne possèdent pas de motif LPXTG ou des répétitions liant la choline. Les mécanismes utilisés par ce dernier type de protéine pour être exporté à la surface de la bactérie sont encore inconnus.

Comme *S. suis* est un pathogène extracellulaire qui adhère probablement aux composants de la matrice extracellulaire (ECM) lors des différents étapes de la pathogénèse, les principaux objectifs de ce travail étaient : i) d'étudier l'adhésion de *S. suis* sérotype 2 aux plus importants composants de la ECM, ii) d'identifier et de caractériser les adhésines impliquées dans l'adhésion de *S. suis* sérotype 2 aux protéines de l'ECM, plus particulièrement à la fibronectine, iii) de déterminer le rôle de ces adhésines dans la pathogénèse de l'infection causée par *S. suis* sérotype 2 et finalement, iv) d'étudier l'activité protectrice de ces adhésines.

Nous démontrons pour la première fois que *S. suis* sérotype 2 peut lier spécifiquement les composantes majeures de l'ECM, comme la fibronectine et différents types de collagène. Alors que *S. suis* ne peut lier directement le collagène de type IV des membranes basales, il a le potentiel d'adhérer à cette protéine par un nouveau mécanisme en utilisant la fibronectine liée à sa surface comme pont. Cette propriété pourrait s'avérer être un nouveau mécanisme de colonisation et d'évasion immune de *S. suis*. La forte inhibition de l'adhésion de *S. suis* à la fibronectine et au collagène lorsque traité aux protéases et à la chaleur indique que ces mécanismes de liaison sont médiées surtout par des protéines. De plus, ces travaux démontrent que l'adhésion de *S. suis* à la fibronectine et au collagène est principalement de type sortase-indépendant, indiquant que la plupart des adhésines impliquées dans ces adhésions, contrairement à ce qui a été préalablement décrit pour d'autres streptocoques, ne sont pas des adhésines de la famille MSCRAMM.

Lors de nos efforts pour mieux comprendre la pathogénèse de l'infection causée par *S. suis*, nous avons identifié et caractérisé une nouvelle protéine liant la fibronectine. Cette protéine a été identifiée comme étant une α-énolase (SsEno). La séquence en acides aminés de cette protéine démontre une absence de motif LPXTG ou de répétition liant la choline, classant cette protéine dans le nouveau groupe désigné protéines « anchorless » et attestant que l'activité des protéines liant la fibronectine de *S. suis* n'est pas reliée aux MSCRAMMs. À ce jour, la principale fonction décrite pour les énolases bactériennes de surface est une activité de liaison au plasminogène. Par contre, nous avons décrit pour la première fois que l'énolase de surface de *S. suis* n'a pas seulement une grande affinité pour le plasminogène mais aussi une affinité semblable pour la fibronectine. De plus, nous avons montré que cette protéine multifonctionnelle est aussi une protéine de choc thermique qui a aussi une activité de liaison aux IgG. Fonctionellement, nous avons prouvé que cette protéine est une nouvelle adhésine de *S. suis* qui participe à la capacité d'adhésion et d'invasion de cette bactérie via-à-vis les cellules endothéliales, indiquant que SsEno pourrait être une molécule clé dans la pathogénèse de l'infection causée par ce pathogène. Ces caractéristiques, incluant la grande immunogénicité de cette protéine chez le porc, fait de SsEno un candidat intéressant pour un vaccin contre les infections à *S. suis*. Par contre, les résultats obtenus dans ces travaux démontrent que, malgré que cette protéine induit une bonne réponse immunitaire, SsEno ne confère pas une protection contre les l'infection à *S. suis*.

En resumé, ces travaux ont permis d'identifier une nouvelle adhésine importante de *S. suis* qui pourrait participer dans différentes étapes de la pathogenèse de cette bactérie comme l'évasion par la bactérie du système immunitaire de l'hôte et l'entrée de la bactérie dans le système nerveux central par les cellules endothéliales formant la barrière hémato-encéphalique.

Mots clés: *S. suis*, MEC, fibronectine, enolase, IgG, HSP, adhésion, invasion, cellules endothéliales, vaccin

SUMMARY

Streptococcus suis is an important bacterial pathogen of pigs that mainly causes septicemia, meningitis, endocarditis, and arthritis. Of the 35 known serotypes, serotype 2 is the most frequently isolated and associated with disease. *S. suis* is also a zoonotic agent. Humans can be infected with the pathogen while handling infected pig carcasses and meat through exposed cuts and abrasions on their hands. Human infections may be severe, with meningitis, septicemia, endocarditis, and deafness as possible outcomes of infection. Death can also occur.

The pathogenesis of the infection caused by *S. suis* is not clear and many steps are probably involved. The mechanisms that enable *S. suis* to disseminate from the tonsils (animal reservoir) throughout the animal are not well understood. It is commonly believed that the ability of bacteria to adhere to host tissues is a critical step in the onset of most microbial infections. This adhesion is the first step for the colonization of mucosal surfaces and may also be the first step before the invasion of host cells, a process that may lead to bacteremia and sepsis. One of the most known and studied bacterial adhesins are the fibronectin-binding proteins. In addition, most of the fibronectin-binding proteins described up to now for different pathogens have been suggested as potential vaccine targets for preventing bacterial infections since antibodies against these surface proteins are believed to have the dual activity of both adherence blocking and opsonic function.

Up to now, three types of streptococcal fibronectin-binding proteins have been identified: i) the MSCRAMM family, containing an LPXTG motif that mediates bacterial surface binding by sortases, ii) the choline-binding proteins that lack the LPXTG motif but contained choline-binding repeats which interact with the phosphorylcholine of the lipoteichoic acid of Gram positive bacteria, and finally iii) the new denominated "anchorless" proteins which do not possess LPXTG motif or choline-binding repeats. The mechanism used by this type of proteins to be exposed to the surface of bacteria is still unknown.

As *S. suis* is primarily an extracellular pathogen and it presumably adheres to components of the extracellular matrix (ECM) to initiate an infection, the main objectives of this work were: i) to study the adhesion of *S. suis* serotype 2 to some of the most important ECM proteins, ii) to identify and characterize the adhesins involved in the adhesion of *S. suis* serotype 2 to the ECM proteins, specially to fibronectin, iii) to determine the role of these adhesins in the pathogenesis of the infection caused by *S. suis* serotype 2 and finally, iv) to study the protective activity of these adhesins.

We have demonstrated for the first time that *S. suis* is able to specifically bind to major constituents of the ECM such as fibronectin and different types of collagen. Interestingly, while *S. suis* is not able to bind directly to basement membrane collagen type IV, it has the potential to

adhere to this protein via a surface-bound fibronectin mechanism. This property might represent a novel mechanism of colonization and immune evasion. The dramatic reduction in binding observed after protease and heat-treatment of *S. suis* to both, fibronectin and collagen, indicates a protein-mediated binding mechanism for these adhesions. In addition, this work also demonstrates that the adhesion of *S. suis* to fibronectin and collagen is mostly sortase-independent, which indicates that most of the adhesins involved in these *S. suis* adhesions, in contrast with what it has been described early for other streptococci, are not adhesins from the MSCRAMM family.

In our continued effort to understand the pathogenesis of the infection caused by *S. suis*, we have identified and characterized a predominant *S. suis* fibronectin-binding protein identified as an α-enolase (SsEno). Its amino acid sequence shows the absence of LPXTG motif or choline-binding repeats, including this protein in the new denominated "anchorless proteins" and corroborating that the fibronectin-binding activity of *S. suis* are not related with MSCRAMMs. Up to now, the major function described for surface bacterial enolases is a strong plasminogen-binding activity. However, we have demonstrated for the first time that *S. suis* surface enolases have not only a high affinity for plasminogen but also a similar high affinity for fibronectin. In addition, the multifunctional SsEno is a heat-shock protein that also has IgG-binding activity. We have also shown that this protein is a new *S. suis* adhesin that participates in the adhesion to and invasion of this bacterium to endothelial cells, indicating that SsEno could be a key molecule in the pathogenesis of the infection caused by this pathogen. These features, including the high immunogenity of this protein in pigs, made of SsEno an interesting candidate for a vaccine against *S. suis* infections. However, results obtained in this work demonstrated that, although this protein elicits a good immune response, SsEno did not confer protection against experimental *S. suis* infection.

In conclusion, this work has permitted to identify a new and important *S. suis* adhesin which can participate in the different steps of the pathogenesis of the infection caused by this bacteria including its evasion of the host immune system and its entry to the CNS through the microvascular endothelial cells forming the blood brain barrier.

Key words: *S. suis*, ECM, fibronectin, enolase, IgG, HSP, adhesion, invasion, endothelial cells, vaccine

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LIST OF ABBREVIATIONS

°C:	centigrade degrees
A:	adenine
aa:	amino acid
Abs:	antibodies
AD:	arginine deiminase
ADP:	adenosine 5'-biphosphate
ADS:	arginine deiminase system
Ala:	alanine
ANCA:	anti-neutrophils cytoplasm antibodies
Arg:	arginine
Asn:	asparagine
Asp:	aspartic acid
ATP:	adenosine 5'-triphosphate
BBB:	blood brain barrier
BC:	before Christ
BMEC:	brain microvascular endothelial cells
bp:	base pairs
BSA:	bovine serum albumin
C.F.U.:	colony forming units
C:	cytosine
CAM:	cell adhesion molecules
CAR:	cancer-associated retinopathy
CCF:	cerebrospinal fluid
Cd^{2+}:	cadmium
CK:	carbamate kinase
CNS:	central nervous system
Co^{2-}:	cobalt
CPS:	polysaccharide capsule
DPP IV:	dipeptidyl peptidase IV
EACA :	ε-amino-n-caproic acid
Eap:	extracellular adherence protein
ECM:	extracellular matrix
EDTA:	ethylenediaminetetraacetic acid

EF:	extracellular factor
EGF:	epidermal growth factor
ELISA:	enzyme-linked immunosorbent assay
FBPS:	fibronectin-fibrinogen binding protein
$Fe(II)^{2+}$:	ferrous iron
G:	guanine
GAG:	glycosaminoglycan
Gal:	galactose
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
GbO_3:	trihexosylceramide
GbO_4:	globoside
GbO_5:	Forssman glycolipid
GBS:	group B streptococcus
gdh:	glutamate dehydrogenase
Glc:	glucose
Gln:	glutamine
Glu:	glutamic acid
Gly:	glycine
h:	hours
H_2O:	water
H_2O_2:	hydrogen peroxide
HA:	hyaluronic acid
HAP:	cell associated stress protein
HE :	Hashimoto's encephalopathy
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His:	histidine
HSP:	heat shock protein
HSR:	heat shock resistant
hyl:	hyaluronate lyase gene
Hyp:	hydroxyproline
ICAM:	intercellular cell adhesion molecule
Ig:	immunoglobulin
IL:	interleukin
ivs:	*in vivo* selected genes
ka:	association constant
kb:	kilobases

kd:	dissociation constant
kDa:	kiloDalton
L:	litre
LPXTG:	Leu-Pro-X-Thr-Gly
LTA:	lipoteichoic acid
Lys:	lysine
MBP:	Myc-binding protein
MC:	mixed cryoglobulinemia
MCP:	monocyte chemotactic protein
Mg^{+2}:	magnesium
mg:	milligram
MHC:	major histocompatibility complex
MLE:	muconate lactonizing enzyme
mm:	millimetres
MMP:	matrix metalloproteinase
Mn^{2+}:	manganese
MR:	mandelate racemase
MRP:	muramidase-released protein
MSCRAMM:	microbial surface components recognizing adhesive matrix molecules
NAD:	nicotinamide adenine dinucleotide
NADH:	nicotinamide adenine dinucleotide (reduced form)
Ni^{2+}:	nickel
nm:	nanometres
O_2^-:	oxygen
OCT:	ornithine carbamoyl-transferase
OFS:	opacity factor of *S. suis*
ORF:	open reading frame
p.i.:	post-infection
PAI:	plasminogen activator inhibitor
PBS:	phosphate-buffered saline
PBST:	PBS containing 0.05% (v/v) Tween 20
PCR:	polymerase chain reaction
PEP:	phosphoenolpyruvate
PGA:	2-phospho-D-glycerate
Phe:	phenylalanine

Pro:	proline
Pyr:	pyruvate
RA:	rheumatoid arthritis
RGD:	arginine-glycine-asparagine
RNA:	ribonucleic acid
Sao:	surface antigen one
SDS-PAGE:	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEN:	*Streptococcus pyogenes* enolase
Ser:	serine
SLE :	systemic lupus erythematosus
sly:	suilysin gene
Sm^{3+}:	samarium
SNC:	système nerveux central
SOD:	superoxide dismutase
SOF:	serum opacity factor
SPR:	surface plasmon resonance
srt:	sortase
SSc:	systemic sclerosis
SsEno:	*Streptococcus suis* enolase
T:	timine
Tb^{3+}:	terbium
TGF:	transforming growth factor
THB:	Todd-Hewitt broth
Thr:	threonine
TLR:	toll-like receptor
TNF:	tumor necrosis factor
tPA:	tissue plasminogen activator
TSP:	thrombospondin
UK:	United Kingdom
USA:	United Stated of America
v/v:	volume/volume
Val:	valine
VIDO:	Vaccine and Infectious Disease Organization
Zn^{2+}:	Zinc
α:	alpha

β:	beta
δ:	delta
ε:	epsilon
γ:	gamma
μg:	microgram
τ:	tau

DEDICATION

Quizá porque mi niñez sigue jugando en tu playa
Y escondido tras las cañas duerme mi primer amor
Llevo tu luz y tu olor por donde quiera que vaya
Y amontonado en tu arena guardo amor, juegos y penas

Yo que en la piel tengo el sabor amargo del llanto eterno
Que han vertido en ti cien pueblos, de Algeciras a Estambul
Para que pintes de azul sus largas noches de invierno
A fuerza de desventuras tu alma es profunda y oscura

A tus atardeceres rojos se acostumbraron mis ojos
Como el recodo al camino
Soy cantor, soy embustero
Me gusta el juego y el vino, tengo alma de marinero

¿Qué le voy a hacer si yo nací en el Mediterráneo?
Nací en el Mediterráneo

Y te acercas, y te vas después de besar mi aldea
Jugando con la marea te vas, pensando en volver
Eres como una mujer perfumadita de brea
Que se añora y que se quiere, que se conoce y se teme

Ay... si un día para mi mal viene a buscarme la parca
Empujad al mar mi barca con un levante otoñal
Y dejad que el temporal desguace sus alas blancas
Y a mí enterradme sin duelo entre la playa y el cielo

En la ladera de un monte, más alto que el horizonte
Quiero tener buena vista
Mi cuerpo será camino
Le daré verde a los pinos y amarillo a la genista

Cerca del mar, porque yo nací en el Mediterráneo
Nací en el Mediterráneo
Nací en el Mediterráneo

Mediterráneo, Joan Manuel Serrat

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I. INTRODUCTION

Streptococcus suis is a major swine pathogen that mainly causes septicemia, meningitis, endocarditis, and arthritis. Of the 35 known serotypes, serotype 2 is most frequently isolated and associated with disease. It has been proposed that two serotypes (serotypes 32 and 34) be excluded from *S. suis* species and re-designated as *Streptococcus orisratti*. *S. suis*, especially serotype 2, has also been described as an important zoonotic agent that affects people in close contact with infected pigs or pork-derived products. Indeed, an important number of cases of human disease with a high rate of mortality in China were directly linked to a concurrent outbreak of *S. suis* infection in pigs.

Little is known about *S. suis* virulence factors. The capsule polysaccharide (CPS) is a critical virulence factor given that unencapsulated isogenic mutants are completely avirulent and rapidly cleared from the circulation in pig and mouse infection models. However, non-virulent strains are also encapsulated, indicating that virulence of this pathogen is a multifactorial process. Another critical *S. suis* virulence factor is the new described OFS (opacity factor serum), since experimental infections of piglets with an isogenic *ofs* mutant strain revealed that OFS is necessary for *S. suis* serotype 2 virulence. OFS carries the typical structural elements of MSCRAMMs, thus it has been speculated that OFS functions as an adhesin and, in particular, that the C-terminal repeats of OFS bind fibronectin. However, no detection of any fibronectin-binding activity was found for the recombinant OFS. Other potential virulence factors have also been described in *S. suis*, including a hemolysin (suilysin), a 136-kDa muramidase-released protein (MRP), a 110-kDa extracellular factor (EF) protein, Sao, a hyaluronidase, a superoxide dismutase, various proteases and different adhesins.

The pathogenesis of *S. suis* infection is not fully understood and likely involves many steps. The pathogen is able to spread systemically from the palatine and pharyngeal tonsils, that are both potential portals of entry for *S. suis*, resulting either in general septicemia or infections of specific organs (e.g. endocarditis, meningitis, arthritis), followed frequently by death. However, the mechanisms that enable the pathogen to disseminate throughout the animal and colonize different tissues are not well understood. It has been demonstrated that *S. suis* is able to bind to and, in some cases, invade endothelial and epithelial cells of human and porcine origin. However, the *S. suis* adhesins and the host receptors involved in these interactions are still unknown.

Host extracellular matrix (ECM) proteins are used as cell receptors by many pathogens. The ECM is a stable macromolecular structure underlying epithelial and endothelial cells and surrounding connective tissue cells. Its composition varies among different organs, but the main components are fibronectin, collagen, elastin, laminin and glycosaminoglycans. Pathogen binding to these ECM proteins might have many consequences that influence their pathogenicity. On one hand these adhesions can mask the microbial surface and thereby interfere with antigen presentation and provide an overall immune evasion strategy. On the other, it can serve as a bridge between the bacterium and host cell surface when ECM proteins bind to their natural receptor on host cell

surfaces such as integrins. Adhesion of pathogen to these integrins via ECM proteins is usually the first step in colonization of tissues but also in penetrating into the body through activation of host cell cytoskeleton. In fact, this activation permits bacterial invasion of host cells and can be used by pathogens to cross some host barriers. While it has been demonstrated that various streptococci specifically bind to host ECM and that these interactions play a role in disease pathogenesis, little is known about the ability of *S. suis* to bind to ECM proteins and the adhesins involved in these adhesions. Only a fibronectin-fibrinogen-binding (FBPS) protein has been proposed as a contributing factor in the colonization of organs due to its binding to ECM proteins of host cells. However, interactions between *S. suis* and ECM proteins have never been studied before.

The majority of pigs that have undergone infections caused by *S. suis* develop a solid immunity indicating that the immune response elicited by the infection is protective. This feature is of fundamental importance for the development of an efficient vaccine to eradicate the *S. suis* diseases around the world. Some of the *S. suis* virulence factors described above have been investigated in vitro and in vivo as suitable candidates for a *S. suis* vaccine. However, up to now, all these efforts to prevent pigs and human infections have failed. As bacterial virulence is determined by a wide variety of factors that influence bacterial attachment, penetration into tissue, and the escape from host, different bacterial ECM-binding proteins have been suggested as potential vaccine targets for preventing bacterial infections because antibodies against these surface proteins are believed to have the dual activity of both adherence blocking and opsonic function.

From this knowledge, our work hypothesis is that adhesion of *S. suis* type 2 to ECM proteins represent a key step in the pathogenesis of the infection caused by this pathogen. The result of this adhesion is bacterial dissemination from tonsils to bloodstream and bacterial penetration across some of the host barriers, such as the blood brain barrier (BBB), which is responsible, at least in part, for the development of *S. suis* infections. In addition, we hypothesize that antibodies against the adhesins involved in those adhesions protect host from *S. suis* infections by preventing bacterial attachment to cells and by functioning as opsonic antibodies.

General aim: To study *S. suis* serotype 2 interactions with ECM proteins

Specific objectives:

I. To study the adhesion of *S. suis* serotype 2 to some of the most important ECM proteins

II. To identify and characterize the adhesins involved in the adhesion of *S. suis* serotype 2 to the ECM proteins

III. To determine the role of these adhesins in the pathogenesis of the infection caused by *S. suis* serotype 2

IV. To study the role of these adhesins as a potential vaccine candidates

II. LITERATURE REVIEW

1. Streptococcus suis

1.1. Introduction:

Streptococcus suis is a major porcine pathogen worldwide. It is one of the most important agents of swine meningitis (152). In addition, it is also responsible of other important diseases in pigs such as meningo-encephalitis, septicemia, arthritis, endocarditis, pericarditis, polyserositis, rhinitis, and abortion (152). *S. suis* is also considered as an opportunistic pathogen or a secondary invader in pneumonia cases because it is commonly isolated from the respiratory tract of sick pigs in combination with other recognized respiratory pathogens (116, 151). Furthermore, *S. suis* is a zoonotic agent related with cases of human meningitis, endocarditis, septicemia and toxic-shock-like syndrome (152). *S. suis* infection in humans is considered as an occupational disease as most of the infected persons were in close contact with infected pigs or with infected carcasses (152).

Biologically, *S. suis* is a Gram-positive bacteria, motionless, ovoid coccus, that can stay singly, in pairs or, in some occasions, in short chains (124). All the known strains are α-hemolytic on sheep blood agar, and many strains can also produce β-hemolysis on horse blood agar (353). Biochemically, *S. suis* is a chemo-organotroph microorganism, with a fermentative and facultative anerobic metabolism (184).

1.2. History:

The first case of *S. suis* infection was reported in Netherland in 1951 by Jansen and Van Dorssen (171). Bacterial isolation in the affected piglets (1-6 months old) demonstrated that these animals carried hemolytic streptococci bacteria in brain and other internal organs (171). Some years later, in 1954, Field *et al.* observed a similar process in which the affected animals were not only piglets but also adult pigs (103). Isolation of bacteria demonstrated that the pathogens responsible were also alpha-hemolytic streptococci. In 1963, De Moor described similar alpha-hemolytic streptococci, also isolated of septicemic pigs, which were different biochemically and serologically from the streptococcal species described at that moment (80). Erroneously, he placed the identified strains in the new serologic groups R, S, RS and T, following the nomenclature of Lancefield (80). Three years later, Elliott suggested that De Moor's group S was similar to his pyomiositis (PM) *Streptococcus* which possessed the wall antigen of the group D of Lancefield, the lipoteicoic acid, and he proposed the name *Streptococcus suis* serotype 1 for this new species (92). In 1975 Windsor and Elliott isolated other porcine streptococci which corresponded to De Moor's group R. They named these bacteria *S. suis* serotype 2 (394). Isolates reacting with antisera against both serotypes 1 and 2 were designated as serotype 1/2, which corresponded to the originally denominated RS

group by De Moor. Between 1983 and 1995, 32 new serotypes based on capsular antigens were described, out of a total number of 35 serotypes (123, 124, 149, 284).

The first time that *S. suis* was officially described as a new species was in 1987 by Kilpper-Bälz and Scheleifer (184). They demonstrated, using hybridation studies, that the serotypes of this new species have genetic homogeneity although they are genetically unrelated to other members of the group D of Lancefield.

The phylogenetic diversity of *S. suis* serotypes was studied later by two independent research groups using the comparison of 16 rRNA gene sequence (63) and a variable region of the chaperonin 60 gene (52). Results showed that 32 of 35 reference strains had a nucleotide sequence similarity which ranged between 93 and 100%, and fell into a major group comprising three clusters. Comparison with nucleotide sequence from other streptococci indicated that, with the exception of serotypes 32, 33 and 34, *S. suis* reference strains did not cluster with any other *Streptococcus* species in the genus. It has recently been proposed that serotypes 32 and 34 be excluded from *S. suis* species and re-designated as *Streptococcus orisratti* (154). However, there is no indication suggesting that members of serotype 33 should be transferred to another species.

The number of untypeable isolates is, in general, relatively low. Most of the times, these isolates are recovered from sporadic cases of disease and it seems that there is no justification at the present time for the characterisation of new capsular types (147).

1.3. Distribution

Among the 35 serotypes described, the serotype 2 has always been considered the most virulent and the most frequently isolated serotype from diseased animals (128). However, the situation may be different depending the geographical location and also, throughout time. For example, the percentage of *S. suis* serotype 2 strains isolated from diseased animals in Canada decreased in the last years and remained relatively low compared with those reported in some European countries, such as France, Italy and Spain, where most of isolates recovered from diseased animals belong to serotype 2 (39, 396). Under specific circumstances, some strains belonging to other serotypes of *S. suis* appear highly virulent, as it is the case for serotype 14 in UK (145), serotypes 1/2 (unpublished observations M.Gottschalk) and 5 (71) in Canada and serotype 9 in central Europe (396).

Serotype 2 is considered the main cause of serious infections in humans, especially in people in close contact with swine or pork products. *S. suis* does not usually cause outbreaks of human infection. However, after the first *S. suis* human case described in Denmark in 1968 (20), sporadic cases have been reported in many countries that have intensive swine industry. To date, most of the human infections have occurred in northern Europe and Asia (223). The total number of cases worldwide is well over 400 (223). China, Thailand, and the Netherlands are responsible for

69%, 11% and 8% of the total cases reported, respectively (223). Mysteriously, only few cases have been reported in Canada and USA. This is probably the consequence of a serious diagnostic problem in laboratories working with human medicine. In fact, most of these laboratories would probably misidentify an isolate of *S. suis* as enterococci, *Streptococcus pneumoniae*, *Streptococcus bovis*, viridans group streptococci or even *Listeria spp.* (152). In many cases, the initial Gram stain diagnosis of the cerebrospinal fluid (CSF) specimen is considered as pneumococcal meningitis.

1.4. *S. suis* infections

Streptococcus suis is a well-recognized worldwide swine pathogen of emerging clinical significance in most countries with intensive swine industry. However, important cases of *S. suis* infections in humans have also been reported in the last years.

1.4.1: In swine

The first *S. suis* case reported in pigs was in Netherlands in the 50's (171). After that, it has been observed that *S. suis* is a worldwide cause of a variety of porcine infections being the most commons septicemia, arthritis, endocarditis, meningitis, pericarditis, rhinitis and abortion, among others (152).

Although *S. suis* is commonly isolated from the respiratory tract of pigs, it is unclear if it is also responsible of another illness like pneumonia because *S. suis* is usually isolated in sick pigs with other recognized respiratory pathogen like *Pasteurella multocida, Actinobacillus pleuropneumoniae, Haemophilus parasuis, Bordetella brochiseptica* and others. Thus, it is commonly believed that *S. suis* could behave as an opportunistic pathogen when other pulmonary pathogens are found in lungs (384). However, some controversy appears when pure cultures of *S. suis* are isolated in cases of swine pneumonia (116, 151). In contrast, in cases of meningitis, *S. suis* is the only microorganism recovered from the brain, and thus is considered a primary pathogen (90).

1.4.1.1. Clinical signs

The earliest sign of *S. suis* infection is usually a rise in rectal temperature to as high as 42.5°C (152). This may occur at the beginning without any other obvious clinical sign. This high temperature is accompanied by a detectable bacteremia or pronounced septicemia (152). If pigs don't receive any treatment, these symptoms may persist for up to 3 weeks. During this period, there is usually fluctuation in body temperature and variable degrees of inappetence, depression, and shifting lameness (68). In some cases, pigs may be found dead without any premonitory signs (152). In cases of septicemia, *S. suis* is isolated from a great variety of organs like spleen, liver, heart, lung and brain where there are inflammatory lesions, without a typical pattern (302). Infected pigs that resist septicemia generally have gross lesions in the central nervous or respiratory system, but not in both (302). In cases of meningitis, the most consistent clinical signs are the neurological

ones like lateral recumbence, paddling, convulsions, ataxia and opisthotonus (353). The predominant lesions found in a pig infected with this pathogen are neutrophilic meningitis and chorioiditis, with hyperemic meningeal blood vessels, fibrinopurulent or suppurative epicarditis and suppurative bronchopneumonia (98, 302, 321). Evidence of encephalitis, oedema and congestion of the brain may also be present (353). In addition, the choroid plexus may have disruption of the plexus brush border and fibrin and inflammatory cell exudates may be present in the ventricles (353). These microscopic lesions do not seem to be serotype-associated (302).

Some of the pigs with the acute form of the disease survive, resulting in healthy carriers or alternatively the infection becomes chronic. In chronic disease lameness and residual nervous signs such as otitis interna might be evident, as well as chronic arthritis signs (225, 302).

1.4.1.2. Transmission

The main *S. suis* reservoirs seems to be the swine (152). The most common tissues colonized by *S. suis* in healthy pigs are the tonsils and nasal cavities, from where it can disseminate throughout the animal without exhibiting any clinical feature (24, 71, 85, 155). Different carrier rates from 0 up to 100% in the upper respiratory tract have been reported (19, 69, 255). This carrier status in tonsils might persist even after treatment with penicillin (353). Some carrier animals will remain healthy carriers, whilst other, will sooner or later develop clinical signs (128). Pigs of any age can be infected with *S. suis*, but susceptibility generally decreases with age following weaning (152). Outbreaks are usually attributed to the introduction of a carrier into the herd (152). However, within a carrier herd, outbreaks can occur especially at young and stress predisposed animals (152).

Several ways for the transmission of *S. suis* between animals in a herd have been suggested: i) piglets born to sows with genital colonization (13), ii) respiratory transmission via aerosols by nose to nose contact (38), iii) oral transmission by contact with a source of infection and, iv) transmission via the nasal, genital or alimentary tract (353).

It has been demonstrated that faeces, dust, water and feed may become secondary sources of infection in which bacteria can persist for short or long periods (353). In addition, vectors of *S. suis* such as flies and mice can also play a role in disease transmission (97).

1.4.1.3. Diagnosis

Diagnosis of *S. suis* infections is generally based on i) clinical signs, ii) age of animals and iii) macroscopic lesions (152). Confirmation of the *S. suis* infections is achieved by i) the isolation of the infectious agent and ii) the typical microscopic lesions in tissues (152).

Since *S. suis* is a normal inhabitant of the upper respiratory tract of pigs and may be recovered from lungs of healthy pigs, it is important to isolate this pathogen in other tissues, overcoat in case of septicemia, because, as mentioned above, it is important to interpret with caution the presence of *S. suis* in the respiratory tissues. Once isolated, identification of bacterial isolates as *S. suis* can be done by different tests (see point 1.5)

1.4.1.4. Treatment

The choice of the best antibacterial agent against *S. suis* infections must be based on several criteria such as i) the susceptibility of the organism, ii) the type of infection and iii) the mode of administration (152). In fact, it has been demonstrated that *S. suis* is highly susceptible to several antibiotics.

A study in which the antimicrobial susceptibility of *S. suis* strains isolated from diseased pigs to 10 compounds licensed in veterinary medicine was carried out recently in Europe (398). The antimicrobial agents included in the tests were ceftiofur, cefquinome, enrofloxacin, florfenicol, gentamicin, penicillin, spectinomycin, tetracycline, tilmicosin and trimethoprim/sulphamethoxazole. The isolates that were tested were collected in seven European countries and represent the most common serotypes involved in clinical disease (396). Results showed that 100% of the strains tested were sensible to ceftiofur, cefquinome, enrofloxacin, florfenicol and penicillin. Only 1.3% of the strains were found to be resistant to gentamicin, 3.6% to spectinomycin and 6% to trimethoprim-sulphamethoxazole. In contrast, high levels of resistance were observed for tetracycline (75.1%) and tilmicosin (55.3%) (398). It is important to remark that variations in antimicrobial usage from one country to another, variations in methodology and differences in serotypes tested may contribute to apparent differences in antimicrobial susceptibility within the countries. These results suggest that we have to be careful in the treatment of *S. suis* infections and that antibiotics have to be used only in cases where sensibility tests have shown that *S. suis* is sensitive (152).

Up to now, the prompt recognition of the early clinical signs of streptococcal meningitis followed by immediate parenteral treatment of affected pigs with an appropriate antibiotic is currently the best method to maximize pig survival (353). In addition, adjunctive therapy with an anti-inflammatory agent is recommended for treatment of *S. suis* meningitis in pigs (13). Treatment can also be administered via the drinking water or in medication feed. However, due to the spread features of the disease, treatment needs to be started very quickly and as it has been explained, pigs in early stage of infection may be difficult to detect (152).

1.4.1.5. Prognosis

Although morbidity rarely exceeds 5%, it can reach more than 50% in cases of poor hygiene and/or concurrent disease (353). Once a herd is infected, mortality is usually low (0-5%) with appropriate treatment at the beginning of the infection. However, it can approach 20% in untreated or wrongly treated herds (71).

1.4.2: In humans

The first cases of severe human meningitis and sepsis due to *S. suis* capsular type 2 occurred between 1968 in Denmark (20). Since these cases, many other cases have occurred around the world in the most diverse places like in the Netherlands, Denmark, Italy, the United Kingdom,

France, Spain, Portugal Sweden, Ireland, Austria, Hungary, Hong Kong, Japan, Argentina, Belgium, Germany, Taiwan, Canada, Singapore, Croatia, Thailand and China (152). Except for two human *S. suis* infection cases caused by serotype 1 (although not serologically confirmed), two cases of septicemia caused by serotype 14 and one case caused by serotype 4 all other human *S. suis* infections are attributed to serotype 2 (190, 389).

In Hong Kong and Vietnam, *S. suis* has been identified as one of the most common causes of meningitis in adults (162, 229). In Canada, there are not any recorded cases earlier than 1982, and the first case in Quebec was diagnosed in 1996 (243). In China, an outbreak of the disease in humans caused by *S. suis* serotype 2 with higher than usual human morbidity and mortality was reported in Sichuan province in 2005 (223). During the Sichuan outbreak, a total of 204 human cases, with 38 fatalities, have been reported (223).

1.4.2.1. Clinical signs

Human infections with *S. suis* are most frequently manifested as purulent meningitis, but cases of septic shock with multiple organ failure, endocarditis, pneumonia, arthritis, and peritonitis have also been reported (223). Differences in clinical signs among patients infected with *S. suis* have been observed. In the acute form of meningitis, symptoms include high fever, headache, chills, nausea, vomiting, and vertigo, followed by one or more of the following: hearing loss, walking ataxia, coma, neck stiffness, petechia, articular pain, peripheral and facial paralysis, severe myalgia, ecchymosis, rashes, and rhabdomyolysis (110, 233, 311, 362). In the acute form of toxic septic shock, besides high fever, chills, headache, vomiting, vertigo, and abdominal pain, other clinical signs were also observed, such as hypotension, tachycardia, liver dysfunction, subcutaneous hemorrhage (*purpura fulminans*), disseminated intravascular coagulation, acute renal failure, and acute respiratory distress syndrome (110, 236, 389). Hearing loss is the most common sequela after recovery from purulent meningitis, whereas death often follows septic shock (223).

1.4.2.2. Transmission

Most of the infected persons are adults in good health who have had direct contact with pigs and/or their excrements (113). That indicates that *S. suis* infection in human can be considered as an occupational disease (223). It has been proposed that high exposure to *S. suis* may lead to a colonization of the upper respiratory tract without producing any health consequences. Only in some cases, predisposing factors such as splenectomy, alcoholism, diabetes mellitus and malignancy may influence disease progression (118, 161, 389).

Although *S. suis* can colonise human upper respiratory tract, the route of entry used for this pathogen in most of the cases is the skin (243). *S. suis* can use some small injury or minor abrasions as a route of entry and to disseminate to the different organs producing the associated diseases described above (243). However, other cases were reported where the route of entry is not designated because the person who contracted the infection had never been in direct contact with

pigs (152). There are some reported cases where the illness is related to raw pork or uncooked pig's blood consumption, and the route of entry could be the oral or respiratory ones, but this hypothesis has not been demonstrated yet (110).

1.4.2.3. Diagnosis

As for pigs, presumptive diagnosis of human *S. suis* infection is based on clinical signs and microscopic lesions. Confirmation of infection is achieved by isolation of the infectious agent and the recognition of microscopic lesions in tissues. Epidemiology history, such as information about direct contact with sick pigs, is very useful for final confirmation. In addition to clinical information, the initial routine laboratory examination is essential for diagnosis of this disease (236, 311).

Patients infected with *S. suis* display elevated white blood cell counts at $13.8–26.6\times10^9$/L (81–95% neutrophils) and high C-reactive protein concentrations of 130–236 mg/L (normal value <10 mg/L) (236, 311). In some cases, high activities of alanine aminotransferase and aspartate aminotransferase were detected because of liver damage (236). Examination of patient's CSF usually revealed turbidity, accompanied by polymorphonuclear pleocytosis (white blood cell count of $1.25–3.24\times10^9$/L), and very low concentrations of protein and glucose (236, 311). Gram stain of CSF, blood, and sometimes joint fluid, can show pairs or short chains of Gram-positive coccoids.

1.4.2.4. Treatment

As explained before, *S. suis* is sensitive to many antibiotics. Once the infectious agent of *S suis* is verified, antibiotic treatment, accompanied with other associated treatments, is very effective in humans (223). Therapeutic treatment varies between patients, depending on the clinical signs. After confirmation of infection, patients are commonly treated with penicillin G, accompanied by one or more other antibiotics including ceftriaxone, gentamicin, chloramphenicol, and ampicillin (223).

Additional intensive supportive care and treatments, such as maintenance of blood glucose concentrations at 4–6 mmol/L, selective digestive-tract decontamination, strategies for prevention of iatrogenic infection, and intravenous immunoglobulin against shock were required (14).

1.4.2.5. Prognosis

In general, most of the cases in humans have a positive prognosis with an early diagnostic of the disease and the appropriate treatment. However, septic shock syndrome may lead to severe damage of organs, including liver, kidneys, and circulatory system, and therefore, mortality can be high (more than 70%) despite adequate treatments (223). It is usual that, as occurs in swine infections, infected persons have a loss (total or partial) of hearing. The incidence of this sequel in this type of meningitis is higher than with others meningitides (20, 225).

1.4.3: In other species

S. suis has also been isolated from a wide range of other animal species such as cows, cats, dogs, horses and birds among others (192). Different serotypes (1/2, 2, 3, 4, 5, 9, 16, 18, 20, 26, 31 and 33) as well as some non typable strains have been isolated from these species. In fact, reference strains from capsular type 20 and 31 were isolated from diseased calves, and the reference strain from serotype 33 from a diseased lamb (63). In all these species, *S. suis* produce similar diseases than in pig specially meningitis, abortion and bronchopneumonia (192). The probability that *S. suis* can be transmissible from domestic pigs to other wild animals such as wild boars has been studied since different cases of wild boars infected with *S. suis* have been reported (30, 153, 192, 311).

1.5. Identification and detection

The first test used for the identification of *S. suis* is the hemolytic test on blood agar. All *S. suis* strains produce narrow zones of α-hemolysis on sheep blood agar plates. In addition, many *S. suis* strains are also able to produce β-hemolysis on horse blood agar plates (333).

Several biochemical tests can also be used to identify *S. suis*. The biochemical identification of *S. suis* has been performed with a number of commercial multitest kits such as the Index and API systems (224, 375), in addition to conventional tests including growth and fermentation in phenol red broth containing 0.1% agar and 1% lactose, trehalose, sorbitol, raffinose, or inulin, hydrolysis of hippurate, and sensitivity to optochin (98).

Various parameters have been proposed for the biochemical identification of *S. suis* including i) growth in 6.5% NaCl (*S. suis* is able to grown in anaerobic or aerobic conditions, but is unable to grow in 6.5 % NaCl solution) (184), ii) acetoin production [the Voges-Proskauer test, that appears to be the most reliable test for differentiating *S. suis* from *S. bovis* (148)], and iii) acid production from trehalose and salicin [*S. suis* is able to produce acid when grown in presence of D-glucose, sucrose, lactose, maltose, salicin, trehalose and inulin (283)] (148).

However, these biochemical characteristics are so variable that identification is often difficult and may require combination of biochemical reactions, followed by confirmative serotyping (90). The latter method is based on capsular polysaccharide antigens by use of one or more of the following techniques: capsular reaction (122-125), capillary precipitation (124) or a co-agglutination test (249). Most laboratories have adopted the coagglutination technique. Since the majority of the typeable strains belong to serotypes 1-8 and 1/2, it is advisable for diagnostic laboratories to only use antisera corresponding to these serotypes and to send the non typeable isolates to a reference laboratory (150, 155). It is important to note that some isolates cross-react with more than one antiserum using the coagglutination or the capsular reaction tests. The most

notable cross-reactions are with antisera against serotypes 1 and 2 which corresponds to strains of serotype ½, and sometimes, some strains of serotype 1 can cross-react with antiserum of serotype 14 (124, 128).

Today, PCR is also a rapid technique used to detect specific serotypes or strains of *S. suis* in animal carriers, or to identify strains obtained from infected or healthy pigs, or even sick human beings for clinical diagnosis or epidemiological studies. PCR based on the *S. suis*-specific 16S ribosomal RNA (rRNA) region and a species-specific probe (serotypes 1-31) targeting 16S rRNA gene can be used to identify *S. suis* strains (46, 299). A PCR procedure based on a 688 bp fragment within the glutamate dehydrogenase gene (gdh) of *S. suis* serotype 2 was also reported to efficiently amplify a specific fragment from all *S. suis* strains tested (265). A multiplex PCR assay was also developed for this purpose (265). Type 2 and 1/2-specific PCR has also been established for detection of *S. suis* type 2 in human infection (233).

Additionally, immunocapture method (125), fluorescent antibody techniques (76, 278), whole-cell antigen-based indirect ELISA (307), and purified capsular polysaccharide antigen-based indirect ELISA (84) are other techniques used to identify this pathogen.

1.6. Virulence factors

As Salyers and Whitt described in 1994, a virulence factor denotes a bacterial product or strategy that contributes to virulence or pathogenicity (318). In the case of *S. suis*, where most of the studies about virulence factors have been carried out with serotype 2, this definition is not so easy applied. There is a lot of controversy between different authors referring to *S. suis* virulence, but all authors agree on one point: there exist two types of *S. suis* capsular type 2 strains, the virulent and the avirulent one. The presence of virulence factors in a strain does not indicate that it is a virulent strain since two strains with the same virulent factors can be totally opposite in his virulence.

Different studies can designate field strain as virulent or avirulent based on i) the animal state: healthy or sick, ii) the presence of virulent factors and iii) the infection experimental model depending on the use of different strains of mice, preinfected piglets or the age of the pigs (152). In addition, it is also true that the results of the experimental infections of pigs with *S. suis* depend on the status of the animal, the volume of the inoculum, the route of infection, and the presence of *S. suis* as a normal microorganism of the upper tract respiratory of the animal previous to the infection (152).

Different studies proposed that the most important candidates to be virulence factors in *S. suis* are the polysaccharide capsule (CPS), the serum opacity factor (OFS), the proteins MRP (muramidase-released protein) and EF (extracellular factor), the hemolysin (suilysin), the IgG binding protein, hemagglutinins, FBPS (fibronectin binding protein) and other proteins briefly mentioned in this review. Recently, a 2-D gel approach combined with mass spectrometry identify

373 proteins probably inolved in the pathogenesis of *S. suis* (173). Most of the identified proteins were located in the cytoplasm and were involved in energy metabolism, protein synthesis, and cellular processes. Among the different protein spots processed, 87 spots representing 77 proteins were successfully identified in which some virulence-associated proteins of *S. suis* were found, including arginine deiminase, ornithine carbamoyl-transferase, carbamate kinase, muramidase-released protein precursor, extracellular factor, and suilysin (173). Enolase and endopeptidase have been proposed as putative virulence-associated factors in this study (173).

1.6.1: The polysaccharide capsule (CPS)

Many microorganisms synthesize exopolysaccharides, this is, polysaccharides excreted outside the microbial cell. In some cases, these polymers, composed of repeating units of simple sugars, remain attached to the outer surface of the bacterium, and then, they are referred to as capsules. Exopolysaccharide production is usually associated with increased virulence as they may function as adhesins, recognition molecules, resistance to antibiotics and by favouring the camouflage of the pathogen against the host immune response. The thickness of the capsule and its polyanionic charge are responsible for the limitation of the entry of different molecules and ions inside the bacteria, protecting them from the exterior (268). On the other hand, the capsule protects bacteria from phagocytosis by reducing opsono-phagocytosis processes mediated by host defence cells (for example, macrophages and neutrophils) (333). Moreover, production of capsular material can prevent the interaction between the C3b component of the complement system and the phagocytic cells (345) and it has been demonstrated that the presence of sialic acid in the capsule is also been associated with the binding of factor H, which prevents deposition of the C3b component on bacterial antigens (298). In addition, the negative charge that it confers to the bacteria can repulse the phagocytic cells which are also negative in charge (12). Furthermore, the limited immunogenicity of many bacterial polysaccharides, the mimicry of host antigens or active induction of T suppressor cells may contribute to the success of encapsulated bacteria as pathogens (64).

S. suis possesses a rigid cell associated polysaccharide capsule, with a molecular weight of 310 kDa, that excludes India ink (284). *S. suis* serotype 1 capsule is composed of five sugars: galactose, glucose, N-acetyl glucosamine, N-acetyl galactosamine and sialic acid. Type 2 capsule composition differs only in one sugar: rhamnose instead of N-acetyl galactosamine (95). As for other bacteria, the sialic acid present in the capsule of *S. suis* has been proposed to be an important sugar involved in the pathogenesis of the infection caused by this bacterium. Arends and Zanen suggested that virulence of *S. suis* serotype 2 could be related to the presence of sialic acid in the capsular material (20). However, further information about the structures and composition of capsules in other serotypes is, so far, unknown.

To better understand the role of the capsule as a virulent factor, the production of non-encapsulated mutant strains of *S. suis* type 2 was done. Studies with these mutants showed that the

capsule is an important virulence factor of *S. suis* since the non-encapsulated isogenic mutants were shown to be avirulent in swine and mouse models of infections (60, 346). Different studies demonstrated that the probable cause for this avirulence is the fact that these mutants are easily phagocytosed *in vitro* by porcine and mouse phagocytes such as monocytes, macrophages and neutrophils (32, 57, 61, 332) which prevent bacterial dissemination and, as consequence, its delivery to the target organs. In fact, it can be affirmed that the presence of the capsule is really necessary for a good infection cycle of the microorganism and for its capacity to cause disease. However, as explained before, the capsule can hide some important adhesins, involved in some steps of the infection, promoting a decrease in the virulence of this pathogen. It has been demonstrated that *S. suis* capsule is up-regulated during growth in intraperitoneal chambers in rats and pigs (61, 297) and also after cultivation in liquid media supplemented with serum (392). This regulation of capsule expression could be a strategy used by *S. suis* to promote infection and seems to represent a common feature among other streptococcal and bacterial species, such as *Streptococcus pyogenes* and *Haemophilus influenzae* (136, 352).

1.6.2. The cell wall

The cell wall of *S. suis* has also been proposed as an important virulence factor. Several studies have been shown that the cell wall or its components such as the lipoteicoich acid (LTA) contribute to exacerbate the host inflammatory response to infection (129, 152). However, the structure and composition of *S. suis* LTA are poorly known. It has been proposed that LTA from *S. suis* may have a backbone structure similar to that of Group A streptococcal teichoic acid, but with differences in the attachment of glucosyl substituents (94). Besides its involvement in inflammation, LTA may also play a direct role in *S. suis* virulence. Indeed, a recent study has shown that inhibition of the adherence of *S. suis* to porcine BMEC can be obtained by pre-incubation of the cells with purified LTA (379). In addition, it has been proposed that *S. suis* may D-alanylate its LTA and that a high ratio of D-alanine to glycerol phosphate in this molecule may be important for the interaction of this pathogen with host cells (105). In our laboratory we have recently generated, by allelic replacement, a *ΔdltA* mutant in a virulent *S. suis serotype 2* field strain and the contribution of lipoteichoic acid (LTA) D-alanylation to the virulence traits of this swine pathogen and zoonotic agent was evaluated. Absence of LTA D-alanylation resulted in increased susceptibility to the action of cationic antimicrobial peptides (107). In addition, and in contrast to the wild type strain, the *ΔdltA* mutant was efficiently killed by porcine neutrophils and showed diminished adherence to and invasion of porcine brain microvascular endothelial cells (107). Finally, the *ΔdltA* mutant was attenuated in both the mouse and porcine models of infection, probably reflecting decreased ability to escape immune clearance mechanisms and impaired

capacity to traverse across the host barriers (107). The results of this work suggest that LTA D-alanylation is an important factor in *S. suis* virulence (107)

1.6.3. MRP and EF

MRP (muramidase-released protein) is a 136 kDa cell wall associated protein, which can also be released to the culture supernatant by bacteria, whereas EF (extracellular factor) is a 110 kDa extracellular protein (382). Up to now, five EF variant genes were identified in serotype 2 strains as well as a smaller (MRP^S) and a larger (MRP*) variants (117, 347, 382, 383). Both proteins were first identified as potential virulent factors of *S. suis* serotype 2, because most of the strains that caused diseases in Europe are MRP and EF positives (354, 381, 382). However, this hypothesis was rapidly discarded when, as observed later, most of the North American virulent strains that produce disease in animals do not express MRP and EF (126).

To the present, although the genes encoding the two proteins were cloned and characterized, their function in the pathogenicity of the *S. suis* infection remains unclear (347, 348). Some findings indicate that MRP could be an adhesin because the anti-MRP serum is able to inhibit the *S. suis* adherence to some cells (363). However, although it has also been shown that a particular region in the MRP aminoacid sequence is very similar to a fibronectin-binding protein of *Staphylococcus aureus*, binding to fibronectin could not be confirmed at present (348). It has been recently demonstrated that *mrp* gene expression levels were dramatically upregulated both *in vitro* and *in vivo* corroborating that *mrp* upregulation in the initial stage of infection may play a role in adhesion (363). In contrast, the *ef* gene functions differently than the *mrp*. In most organs, it was not notably upregulated until 48 or 72 h post infection suggesting that EF may play a different role during infection (363).

To verify the virulence potential of MRP and EF, Smith *et al.* infected newborn pigs with isogenic mutant strains for the expression of both proteins (349). Results showed that mutants that lacked EF and MRP were still as virulent as the wild type strains suggesting that the proteins themselves do not contribute to the pathogenicity of the bacteria. An alternative explanation for these results is that the virulence associated with *S. suis* is a multifactorial process in which particular functions can be fulfilled by redundant or alternative factors, which means that, in absence of MRP and EF, other virulence factors can take over their functions. This hypothesis could explain why some North American strains are virulents although not possessing these proteins.

1.6.4: Hemolysin (Suilysin)

Hemolysin is a form of exotoxin protein produced by bacteria which causes lysis of red blood cells *in vitro*. As explained above, visualization of hemolytic effect of this toxin in red blood cells in agar plates facilitates the categorisation of some pathogenic bacteria such as *S. suis*.

Hemolysin activity is normally associated with pathogenicity and virulence in many bacterial species.

It was in 1994 that Jacob *et al.* identified for the first time a 54 kDa *S. suis* hemolysin (167). One year later, Gottschalk *et al.*, described another *S. suis* hemolysin of 65 kDa (130). Different posterior studies corroborated that these two *S. suis* hemolysins were really the same one and that the difference in the molecular weight was caused by the different techniques used for their purification (130). This *S. suis* hemolysin, named suilysin, belongs to the family of toxins known as thiol-activated toxins or more recently named as antigenically related cholesterol-binding cytolytic toxin (332). This name refers to the fact that these toxins do not bind to membranes that do not contain cholesterol or a closely related sterol (263). In fact, interaction with cholesterol also occurs in the absence of any other lipids, i.e. with the pure sterol in solution or suspension (89), and, as occurs for suilysin (57), leads to inhibition of lytic activity (290). Other hemolysins which have common characteristics with suilysin are pneumolysin (*S. pneumoniae*), listeriolysin (*Listeria spp.*) and streptolysin (*S. pyogenes*). These thiol-activated toxins form discrete, oligomeric pores in membranes containing cholesterol (40). The pores produced by these toxins are exceptionally large, assuming an internal diameter of up to 30 nm and comprising around 50 monomer subunits (40). The ring-shaped, non-covalently bonded oligomers have been visualized by electron microscopy (89) before being functionally characterized as transmembranal pores. In the case of suilysin, the pores formed in membranes are about 7 nm (130).

Suilysin has a cytotoxic effect *in vitro* on epithelial (199, 261), endothelial (62, 378) and immune cells (32, 57, 336), suggesting that this *S. suis* toxin could play a role in damaging and penetration of different cells and tissues and, in consequence, in the pathogenesis of the bacteria. Moreover, suilysin triggers cytokine expression by human and porcine monocytes (221).

To verify the role of suilysin during infection, *sly* isogenic mutants have been produced and tested in animal trials. Different results have been observed. Allen *et al.* reported that *sly* mutant was avirulent in a mouse infection model (10). However they also reported that the same mutant was only slightly attenuated in a porcine model of systematic infection. In addition, Lun *et al.* documented that three suilysin mutants showed no attenuation in a porcine challenge experiments (221). In addition, as for MRP and EF, this toxin is mainly present in European and Asian strains which imply that, by itself, it can not be considered as a critical virulence factor. However, due to the multifactorial process involving *S. suis* pathogenesis, this protein could, in combination with other factors, be considered as important for the development of the diseases produced by this bacterium.

1.6.5. Hemagglutinins

Bacterial adhesins mediate strong and specific adhesion of bacteria that are colonizing epithelia or invading tissues. The oligosaccharide chains of glycoproteins and glycolipids are ideal

receptors for bacterial attachment, and the heterogeneity of oligosaccharide structures and the specificity of bacterial lectin-like adhesins provide bacterial attachment to different cell types of the body. *S. suis* proteins that possess sugar-specific adherence activities for galactose, N-acetylgalactosamine and sialic acid have been identified by inhibition of hemagglutination (196). On one hand, Haataja et *al.* reported that galactose-binding strains of *S. suis* recognise the Galαl-4Gal sequence that is present in the P^I and P^k blood group antigen structures (139). Furthermore, they showed that *S. suis* is able to bound to trihexosylceramide (GbO_3) but not to globoside (GbO_4) or Forssman glycolipid (GbO_5). GbO_3 is expressed on the surface of erythrocytes as well as in many pig and human tissues, and might represent the receptor for galactose binding strains in pig pharyngeal epithelium (139). Until now, only a galactose-binding protein of 18 kDa with an isoelectric point of 6.4 has been identified in *S. suis* (218). This adhesin was subtyped in P_o (which adhesion to galactose is inhibited only by galactose) and P_N (which activity is inhibited by galactose and N-acetyl-galactosamine). These two adhesin variants have the same N-terminal peptide sequence indicating that they are closely related (366). On the other hand, the cell-binding specificity of sialic acid-recognizing strains of *S. suis* was also investigated by Tikkanen *et al.* (366). They demonstrated that treatment of human erythrocytes with sialidase or mild periodate abolished hemagglutination of *S. suis*. Hemagglutination inhibition experiments with sialyl oligosaccharides indicated that *S. suis* possesses an adhesin for this sugar and that the adhesion involved preferred the sequence NeuNAc alpha 2-3Gal beta 1-4Glc (366). However, up to now, the adhesin involved in this process has not been identified.

1.6.6. Albumin-binding protein and GAPDH

The binding capacity of *S. suis* serotype 2 to albumin was also investigated because it has been demonstrated that the adhesion of *S. suis* to this plasma protein increase its virulence (294). This study indicated that a 39-kDa protein was responsible for the interaction with albumin. However, since different surface molecules have been described to possess an albumin binding activity in other streptococci (344), the possibility that other cellular components could also contribute to this activity in *S. suis* cannot be ruled out. This theory was supported by the fact that an avirulent strain that produces a thicker capsular material showed a higher binding to albumin than other virulent strains with thinner capsule (297), suggesting that capsular material could also be involved in this binding activity (294).

Interestingly, posterior genetic studies demonstrated that the N-terminal sequence of this 39-kDa protein of *S. suis* possesses a high degree of homology with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of group A streptococci indicating that in fact, this 39 kDa protein is the GAPDH of *S. suis* (47). Recombinant GAPDH from *S. suis* was found to possess functional GAPDH enzymatic activity after the purification. In addition, adherence assay with *S. suis* and

porcine tracheal rings pre-incubated with this recombinant GAPDH showed a significant reduction in the adhesion of *S. suis* to tracheal rings suggesting that this protein could be involved in the first steps of the bacterial adhesion to host cells (47). To verify the role of this protein in the virulence of *S. suis,* mutants defective in the expression of this protein were constructed and tested for their adhesion to tracheal cells and porcine tracheal rings (47). Results showed a reduction in their binding capacity compared with the wild type strain, indicating that this protein is localized in the surface of the bacteria and that it seems to play an important role in bacterial adhesion. However, the fact that this protein is also present in the non-virulent strains of this pathogen confirms again the multifactorial character of the pathogenesis of this pathogen.

1.6.7: IgG binding protein

Another protein isolated, purified and characterized from *S. suis* serotype 2 as a potential virulence factor is a 52 kDa IgG-binding protein. This IgG-binding protein has been found associated with the cell surface, but it has been demonstrated that it can also be released in a soluble form during bacterial growth (35, 342, 343). In addition, it represents a common antigen found in all strains of *S. suis* serotypes tested, including the non-virulent ones, determining that this factor alone does not participate in virulence (35, 342, 343). The role of this protein is unknown at time. However, its IgG-binding activity suggests that it may play a role in the evasion of the host defenses.

Some years later, studies on the presence of stress or heat shock proteins (HSP) in *S. suis* using polyclonal and monoclonal antibodies directed against different bacterial heat shock proteins identified a new protein with an apparent molecular mass of 60 kDa. As for the 52 kDa IgG-binding protein, this HSP was identified on the surface of all *S. suis* serotypes. Posterior sequence analysis demonstrated that this protein was in fact the identified 52 kDa IgG-binding protein indicating that this protein, in addition of its IgG-binding activity is also member of the 60 kDa heat shock protein family (34, 35). The difference on the molecular weight in both studies was probably due to the different purification methods used.

1.6.8: Fibronectin-fibrinogen-binding protein (FBPS)

Many important virulence factors are environmentally regulated and are induced at specific stages of the infection process (228). To identify these genes in *S. suis*, the group of de Greef *et al.* cloned promoters and their downstream sequences that are "on" during experimental *S. suis* infection of piglets (79). Twenty-two *in vivo*-selected (*ivs*) genes were found (79). Two of the *ivs* genes were directly linked to virulence since homology to genes in the database that encode for known virulence factors was found (79). One of them showed homology to genes encoding fibronectin- and/or fibrinogen-binding proteins of *Streptococcus gordonii* and *S. pyogenes* (73).

The *fbps* gene was shown to be present in all known serotypes of *S. suis* (except serotypes 32 and 34 that, up to date, can be described as *S. orisratti* instead of *S. suis*), as well as in all known

phenotypes of serotype 2. However, the expression of FBPS in all serotypes and phenotypes was not studied indicating that although all strains, except for serotypes 32 and 34, possess the *fbps* gene, maybe not all strains express FBPS.

A considerable number of fibronectin-binding proteins of various bacterial species have been shown to be important virulence factors (177). The role of FBPS in the pathogenesis of *S. suis* was studied in an experimental infection model in piglets. Since the authors were unable to determine a 50% lethal dose for the mutant strains, they decided to compare the virulence of the isogenic FBPS mutant with that of the wild-type *S. suis* strain in a competitive infection assay in piglets (78). Data obtained clearly showed that the mutant strain was capable of colonizing the tonsil as efficiently as the wild-type strain indicating that FBPS is not involved in the colonization of the tonsil (78). However, results also indicated that FBPS does play a role in the colonization of specific organs, since joints and the CNS were more efficiently colonized by wild-type than by mutant bacteria (78). Unfortunately, these authors never evaluated the adhesion capacity of the FBPS mutant to fibronectin. In addition, de Greef *et al.*, also demonstrated that FBPS reacted with a convalescent-phase serum of a pig that survived an *S. suis* infection indicating that FBPS is immunogenic in pigs and corroborating that FBPS of *S. suis* is expressed under *in vivo* conditions (78). In addition, another recent study has demonstrated that *fbps* expression was upregulated in all organs 24 h postinfection indicating that *fbps* could act as an adhesin and that high initial expression levels were critical for virulence (363).

1.6.9. Serum opacity factor

Recently, Baums et *al.*, identified a gene of *S. suis*, named *ofs* (29), which encodes a protein homologous to other streptococcal fibronectin-binding proteins such as FnBA of *Streptococcus dysgalactiae* (237) and SOF of *S. pyogenes* (72) As OFS carries the typical structural elements of MSCRAMMs (see at point 2.3.1), it has previously been speculated that OFS functions as an adhesin and in particular that the C-terminal repeats of OFS bind fibronectin. However, no detection of any fibronectin-binding activity was found for the recombinant OFS (29). The lack of detectable fibronectin-binding might be explained by significant differences between the repetitive sequences of OFS and the fibronectin-binding repeats of FnBA (237) and SOF (29).

On the other hand, it has been demonstrated that, as for SOF of *S. pyogenes* (72), OFS also confers surface-associated serum opacification activity (29). This activity has not been proven to be essential for virulence, and probably, other as-yet-unknown functions of OFS might be involved in pathogenesis (29) since experimental infections of piglets with an isogenic *ofs* mutant strain revealed that OFS is a major virulence determinant in *S. suis* serotype 2 (29). These results are in accordance with the attenuation of virulence in an SOF knockout *S. pyogenes* mutant described previously by Courtney *et al.* (72) and corroborate that, in addition of the CPS, OFS is another proved virulence factor for *S. suis*.

A recent study using PCR screening and sequencing analysis of OFS from 108 *S. suis* isolates from diseased and healthy pigs, and human patients shows that besides the ofs gene reported before (designated type 1), there were three more allelic variants of ofs (designated types 2 to 4) (361). Type-1 and type-2 ofs genes encode functional OFS and as consequence opacified horse serum. In contrast, type-3 ofs was interrupted by a point mutation and type-4 ofs was disrupted by either insertion of an IS element or genetic rearrangement, and therefore did not show serum opacification activity (361). These results suggest that OFS could contribute to the virulence of only some *S. suis* isolates.

1.6.10. Surface antigen one (Sao)

A *S. suis* surface protein reacting with convalescent-phase sera from pigs clinically infected by *S. suis* type 2 was also identified (216). The apparent 110-kDa protein, designated Sao, exhibits typical features of membrane-anchored surface proteins of gram-positive bacteria (see point 2.3.1), such as a signal sequence and an LPXTG membrane anchor motif (216). In spite of high identity with the sequenced genomes of *S. suis* Canadian strain 89/1591 and European strain P1/7, Sao does not share significant homology with other known sequences. However, a conserved avirulence domain that is often found in plant pathogens has been detected (216). A recent characterisation of *S. suis* Sao demonstrate that there are, at least, three allelic variants of sao gene, namely sao-S, sao-M, and sao-L based on the different lengths of the genes (approximately 1.5, approximately 1.7, and approximately 2.0 kb, respectively) (100). These differences were determined to be caused by heterogeneity within the number of C-terminal repeat sequences, which had been seen as a pathogenicity-related domain in the plant pathogen, *Xanthomonas oryzae* (100). Electron microscopy using a Sao-specific antiserum confirmed the surface location of this protein on *S. suis*. In addition, the Sao-specific antibody reacts with cell lysates of 28 of 33 *S. suis* serotypes and 25 of 26 serotype 2 isolates in immunoblots, suggesting that this protein is highly conserved in *S. suis* species (216). However, although the immunization of piglets with recombinant Sao elicits a significant humoral antibody response, the antibody response was not reflected in protection of pigs that are intratracheally challenged with a virulent strain before this work (216).

1.6.11. SalK/SalR

As explained before, recent large-scale outbreak of human *S. suis* serotype 2 epidemic in China characterized by a toxic shock-like syndrome has been described in the last years. Studies of the epidemic strains responsible of these outbreaks revealed the presence of a newly predicted pathogenicity island (PAI). Further bioinformatics analysis identified a unique two-component signal transduction system located in this PAI, which is orthologous to the SalK/SalR regulatory system of *Streptococcus salivarius* (215). Knockout of *sal*KR eliminated the lethality of these strains in experimental infection of piglets (215). Functional complementation of *sal*KR into the isogenic mutant Δ*sal*KR restored its pathogenicity (215). Bactericidal assays demonstrated that the

mutant was more susceptible to polymorphonuclear leukocyte-mediated killing than its parental strain. In addition, colonization experiments showed that the Δ*sal*KR mutant could not colonize any susceptible tissue of piglets when administered alone (215). These findings suggest that SalK/SalR is requisite for the full virulence of ethnic Chinese isolates of highly pathogenic *S. suis* serotype 2, thus providing experimental evidence for the validity of this bioinformatically predicted PAI and confirming that SalK/SalR can be considered as a novel virulence factor of *S. suis*.

1.6.12. Enzymes

1.6.12.1. Hyaluronate lyase

The hyaluronate lyase is a secreted protein that degrades hyaluronic acid (HA) into an unsaturated disaccharide (11). HA is a high-molecular-weight polysaccharide consisting of repeating disaccharide units and is a fundamental component of the extracellular matrix and capsular material (205). Many gram-positive bacteria able to adhere to HA are able to cause infection at the mucosal and skin surfaces of humans and animals (217). It is proposed that a decrease in viscosity due to HA depolymerization increases the permeability of connective tissues, which increases the ability of bacteria to spread and contributes to subsequent virulence (217). Human tissues known to contain HA include the blood, brain, liver, umbilical tissue, and skin (205).

Members of several streptococcal species are known to produce hyaluronate lyase (163, 230), and research indicates that this lyase may be important in pathogenesis. In the case of *S. suis* it has been showed that although the *hyl* locus is present in virtually all *S. suis* isolates, only about 30% of isolates express an active protein (185). The gene, in common with many encoding streptococcal surface proteins, has a variable 5' end evolving by the generation of point mutations, insertions or deletions, and recombination events and a more conserved 3' end (185). Despite these observations suggesting the corresponding protein is subject to host immune surveillance, the lack of activity in many virulent field isolates, often associated with the disruption of the hyaluronidate lyase open reading frame, suggested that hyaluronidate lyase is not an important virulence factor of *S. suis* (185). However, a recent study revealed that although *hyl* mRNA levels were very low *in vitro*, it reaches a significant level *in vivo* which led to hypothesise that it is possible that some host factors are required for *hyl* gene expression and that this protein could be an important virulence factor for *S. suis* (363)

1.6.12.2. Arginine deiminase

In addition to all these potential *S. suis* virulence factors two cell wall-associated proteins of *S. suis* that were induced by a temperature shift from 32°C or 37°C to 42°C have been identified (395). Amino-terminal sequence analysis of the two proteins indicated homologies to an ornithine carbamoyl-transferase (OCT) and to the streptococcal acid glycoprotein from *S. pyogenes*, an arginine deiminase (AD) recently proposed as a putative virulence factor (82, 83). Cloning and sequencing of the respective genes of *S. suis*, *adiS* and *octS*, and their adjacent upstream and

downstream regions revealed that they were clustered together with two additional open reading frames (ORFs). The first ORF (*orf-2*) showed 59.8% homology to a gene encoding a hypothetical cytosolic protein, and the second ORF (*ckS*) showed 70.1% homology to a carbamate kinase of *S. pyogenes*. Thus, it was concluded that the genes *adiS*, *octS*, and *ckS* make up the AD system (ADS) of *S. suis* (395).

The ADS catalyzes the conversion of arginine to ornithine, ammonia, and carbon dioxide and concomitantly generates 1 mol of ATP per mol of arginine consumed and comprises three major enzymes, the AD, the OCT, and the CK. In oral streptococci and *S. pyogenes*, the ADS seems to provide protection against acidic stress by the production of ammonia (55, 82). Furthermore, it has been shown that the AD of *S. pyogenes* is involved in adhesion to and invasion of epithelial cells (82, 232). In the case of *S. suis* it has been proposed that the ADS enables *S. suis* to overcome oxygen and nutrient starvation and to tolerate acidic environments (135). Thus, the ADS might facilitate *S. suis* survival within the different niches of the host and thereby probably contributes to *S. suis* pathogenesis. However, future investigations are needed to clarify the importance of the ADS for *S. suis* infections.

1.6.12.3. Superoxide dismutase

Although *S. suis* is considered a facultative anaerobic metabolism bacteria (184), its metabolism is always fermentative with glycolysis serving as the principal energy-yielding pathway. Despite this, and despite the usual lack of hem proteins by this bacterium, growth under aerobic conditions is always associated with the consumption of oxygen (17). Oxygen consumption is due primarily to the activities of flavoprotein NADH oxidases and, depending on the oxidase, the reduced product may be H_2O, H_2O_2, or O_2^- (51, 365). It has been demonstrated that *S. suis* possesses NADH oxidases capable of reducing oxygen to toxic H_2O, H_2O_2, or O_2^- (258). Cellular damage mediated by these reactive oxygen species can be minimized through the dismutation of O_2^- by means of superoxide dismutase enzyme (SOD) or a high intracellular concentration of manganese. It has been demonstrated that *S. suis* produces a MnSOD (200). The activity of this SOD was influenced by the availability of manganese, and when the organisms were grown under manganese-limited conditions, by the availability of iron; however, the extent to which this is relevant *in vivo* is not known (258).

Different studies demonstrated that *S. suis* appears to be well equipped for its existence as an aerotolerant anaerobe (258). It requires manganese, but not iron, for growth, produces a MnSOD to eliminate O_2^- and reduce cellular damage via Haber-Weiss reactions, and in the absence of catalase, and perhaps peroxidase, may be able to exclude iron to minimize cellular damage associated with H_2O_2 and the Fenton reactions (258). Finally, the lack of an iron requirement may also benefit the organism *in vivo* in that unlike other organisms, it would not be subject to, and

indeed might benefit from, one of the major host defence mechanisms, namely, the withholding of iron from invading pathogens (258).

1.6.12.4. Proteases

Proteases are important virulence factors for a variety of microbial pathogens and may contribute to tissue degradation and perturbation of the host defence system (226). It has been reported that *S. suis* produces four major proteases (arginine aminopeptidase, chymotrypsin-like, caseinase, and dipeptidyl peptidase IV (DPP IV)(176).

The Arg-aminopeptidase of *S. suis* was found to be both extracellular and cell-associated protein and showed a molecular mass of approximately 55 kDa. In bacteria, arginine residues released by the action of Arg-aminopeptidases on peptides may be catabolized via the arginine deiminase pathway resulting in the production of ATP and other essential metabolic precursors as explained before (74). *S. gordonii* has also been reported to produce an extracellular Arg-aminopeptidase (121). It was hypothesized that the Arg-aminopeptidase of *S. gordonii* could cleave the arginine residues at the N-termini of substance P and bradykinin thus inactivating their biological function (121). This inactivation could result in the uncontrolled contraction of smooth muscle at the infection site and a change in local vascular permeability. This phenomenon may also apply to *S. suis* but further studies are needed to evaluate this hypothesis.

A strong cell-associated caseinase activity was also detected in all strains of *S. suis* serotype 2. This caseinase activity may have a nutritional function and can also participate in the maturation of precursors as demonstrated in other bacteria (368). For instance, the caseinase of *Aeromonas salmonicida* participates in the processing of a hemolysin precursor produced by this bacterium (368). Since *S. suis* also produces a hemolysin (167), the caseinase activity may play a similar role in its maturation. However, this caseinase function has not yet been studied for *S. suis*.

A cell-associated chymotrypsin-like activity, belonging to the class of serine proteases, was detected only in virulent European *S. suis* strains as was also noted for the suilysin (hemolysin). Fernandez-Espla *et al.* (102) reported the purification of a cell wall-anchored chymotrypsin-like enzyme from *Streptococcus thermophilus*. Interestingly, this protease shares several characteristics (serine protease, substrate specificity, optimum pH and temperature) with the *S. suis* chymotrypsin-like activity identified in this study. However, the function of this protease is still unknown.

Finally, a DPP IV activity was also found in the culture supernatant and on the cell surface of *S. suis*. The optimum activity observed at pH 8 corresponds to that of other DPP IV and homologues previously characterized (331). Inhibitors specific for DPP IV (diprotin A and DPP IV inhibitor I) were highly effective in inhibiting the DPP IV activity of *S. suis*. This activity is also present in mammals on activated T cells (CD26) and in a soluble form in plasma where it regulates bioactive peptides like neuropeptide Y and cytokines (178, 239). Various species of *Lactococcus* (234), *Lactobacillus* (242) and *Streptococcus* (120, 242) have been found to possess this activity

and the genes have been cloned and characterized. The DPP IV of *Porphyromonas gingivalis* is considered a virulence factor since a mutant lacking this activity did not cause as much damage as the wild-type strain in an animal model (195). However, its role in *S. suis* is unknown.

Further studies are required to investigate the pathogenic role(s) of all these four *S. suis* proteases since up to now the exact function of these proteins is still unknown.

1.7. The pathogenesis of *S. suis* infection

Knowledge on the pathogenesis of *S. suis* infection is still restricted. Studies on this subject are almost limited to *S. suis* serotype 2 and only concern the development of swine meningitis. Knowledge of *S. suis* infections to humans is even more limited than in pigs. However, the recent *S. suis* outbreak in Asia has accelerated the study of the pathogenesis of this bacteria in humans (223).

Many question marks remain unclear (Fig. 1). It seems that *S. suis* infection represent a very complex process in which host, bacterial and environmental factors dictate the course of the interaction. Several steps are believed to be central in the production of disease: i) colonisation and invasion of the respiratory epithelium, ii) blood dissemination and phagocytosis resistance, iv) inflammation and v) penetration of the endothelial barrier of the target tissues (59, 128).

1.7.1. Colonisation and invasion of the respiratory epithelium

How bacteria present at low numbers on mucosal surfaces are able to traverse the first mucosal barriers to develop disease is still unknown. In fact, bacteria would need to breach mucosal epithelia in the upper respiratory tract to reach the bloodstream.

To pass through the cells, *S. suis* has first to adhere to respiratory epithelial barrier. Several studies have shown that *S. suis* is able to adhere to different epithelial cell lines, including those of porcine (261) and human origin (199), to pig tissues such as pharyngeal epithelium (139) or to porcine lung sections (127). To mediate these processes, different structures, such as bacterial adhesins seem to be important, however, the exactly molecules involved are still unknown.

The hypothesis that some adhesins are responsible for this adhesion is corroborated by the fact that adherence of *S. suis* to epithelial cells is considerably reduced in the presence of capsule which can hide adhesins (199) These results suggested that, as occurs with other capsulated bacteria, host cell adherence of *S. suis* depends on the state of encapsulation of bacteria (259, 314). It has been hypothesised that encapsulation is down-modulated early in the infectious process in order to avoid interfering with colonization, but, in contrast, encapsulation is up-modulated during bacteremia, where it appears to confer a selective advantage for the pathogen (352). Although it is known that in *S. suis* expression of the capsule is modulated by environmental signals (33, 297), modulation of capsule expression upon the infectious stage has not been proved yet for *S. suis*.

After adhesion to host epithelial cells, *S. suis* has to invade these cells to cross the epithelial barrier. Some controversy has been observed in the capacity of *S. suis* to invade or not epithelial

cells. Although Lalonde *et al.* published that *S. suis* is not able to invade epithelial cells and Norton *et al.* declared that *S. suis* invade epithelial cells "as a rare event" (261), a more recent study demonstrates that different non-typable strains were able to invade epithelial cells (33). Since these studies have been carried out with cell monolayers, studies using respiratory epithelial cells monolayers in a Trans-well system could finally resolve this controversy.

Another approach that can be used by *S. suis* to cross epithelia barriers is the use of suilysin. As explained above, suilysin is a member of thiol-activated cytolysin family of hemolysins (167). The roles of these toxins in pathogenesis of bacteria are varied and not fully understood. However, it has been demonstrated that they all lyse eukaryotic cells that contain cholesterol. In the case of *S. suis* it has been demonstrated that suilysin expressing *S. suis* strains are able to damage the epithelium using this pore-forming toxin, which was shown to play a cytotoxic effect *in vitro* on a wide range of cell lines (199, 261), and might favour tissue invasion. However, this is not seen with suilysin-negative strains.

S. suis also produces other molecules that could help bacteria to cross these epithelial cells destroying the integrity of the barriers. One of these molecules is the enzyme hyaluronate lyase (11) which is able to degrade HA. HA, also called hyaluronane or hyaluronate, is a non-sulfated glycosaminoglycan distributed widely throughout epithelial tissues. It is one of the main components of the extracellular matrix (ECM) and contributes significantly to cell proliferation and migration. The degradation of HA by *S. suis* hyaluronate lyase could permit the enhancement of invasion and the spread of the bacteria during infection by destruction of the ECM (11). In addition, *S. suis* also possesses plasminogen-binding molecules such as GAPDH (174). This molecule is able to capture plasminogen from host plasma and once converted to plasmin (175) bacteria become armed with the broad substrate spectrum proteolytic potential of plasmin that can be used to facilitate bacterial penetration through biological membranes such as the respiratory epithelium barrier.

Finally, a way to circumvent the epithelial barrier would be the so called "Trojan horse theory". This theory suggested that bacteria are taken up directly in the tonsil by monocytes, allowing the bacteria to travel intracellularly from tonsils to bloodstream (393). However, this theory is not very plausible for *S. suis* because i) *S. suis* is not able to survive for long time inside these cells (348) and ii) it has been shown that, because of the presence of capsule, only a low number of monocytes contain intracellular bacteria and most of the bacteria remain attached extracellularly (334, 393). These results indicate that *S. suis* is probably not able to travel intracellularly, but in contrast it could travel extracellularly (393). This new theory was called "modified Trojan horse theory" and suggested that bacteria are able to cross barriers and disseminate towards bloodstream or target organs not inside the cells but in close association with them (128).

1.7.2. Blood dissemination and phagocytosis resistance

As explained above, the strategies proposed by "Trojan horse" theories might help bacteria not only to cross the epithelial barrier but also to disseminate in blood, and even to reach the target tissues by exploiting the monocytes. However, most studies carried out during the last decade suggest that bacteria migrate in blood as free bacteria since a high level of bacteremia usually precedes the onset of bacterial meningitis (393).

Once in the blood, bacteria have to resist the immune system. The first leukocytes recruited to sites of infection are the neutrophils (386). Neutrophils are a type of white blood cell or leukocyte which form an early line of defence against bacterial infections (386). They are the most numerous type of leukocytes and belong to a group called phagocytes. They are part of the innate immune system and are involved in the inflammatory response (386). Neutrophils are produced in huge numbers in response to infection, trauma or other stimuli. Once in place, they eliminate the invading bacteria and other noxious substances, usually dying in the process themselves. One of the methods they use to kill invaders is called phagocytosis which involves engulfing and digesting the "enemy" cell (386).

It has been demonstrated that neutrophils play an important role in innate immunity against *S. suis* because i) they are detected during the early stages of *S. suis* infection, ii) are significantly increased in pigs infected with a virulent strain, and iii) are usually predominant in lesions caused by this pathogen (317, 320, 350, 381). A recent study carried out in our laboratory corroborated that neutrophils play an important role in host defence against *S. suis* infection since they are able to rapidly phagocyte most of the bacteria in presence of specific antibodies and complement (57). However, under non-immunised serum conditions, the capsule protects bacteria from neutrophil-mediated killing whereas non-encapsulated mutants were efficiently killed under the same conditions. Quessy et al. (297) also showed that an increase in capsular material thickness after *in vivo* bacterial growth is accompanied by increased resistance to killing by swine neutrophils. Both studies corroborate the role of CPS as an important virulence factor of *S. suis*. Since neutrophils are the predominant cell type in swine blood, the ability of CPS to effectively protect *S. suis* from killing by these phagocytes may explain, at least in part, its survival capacity in blood (221). However, some avirulent strains also possess capsule which demonstrate that other factors are also important in the bacterial resistance in blood. It has been demonstrated that virulent strains that express EF, MRP and suilysin are more resistant to phagocytosis and killing by neutrophils that virulent strains that lack these factors (32, 57). Particularly, the lack of suilysin seems to be, at least in part, responsible for the higher susceptibility to killing by leukocytes. The principal reason is that, as established for other cell types, suilysin is also toxic for neutrophils (32, 57). However, it has also been probed that suilysin also interferes with complement which can protect, at least in part, *S. suis* from the immune system (32, 57). It is important to remark that, although well

capsulated strains possessing the mentioned virulence factors are able to resist phagocytosis, these strains are rapidly phagocyted and cleared from the circulation by neutrophils in the presence of complete serum and specific antibodies against this pathogen, confirming the important role of opsonic molecules (complement and antibodies) in the phagocytosis process of these immune system cells.

As explained above, neutrophils have been found to be the first leukocytes at the onset of the disease, but after few hours, neutrophils start to die and other immune system cells consisting of mainly monocytes and lymphocytes take their place in the protection of the host (350). Monocytes are produced by the bone marrow from hematopoietic stem cell precursors called monoblasts. These cells circulate in the bloodstream for about one to three days and then typically move into tissues throughout the body. They constitute between three to eight percent of the leukocytes in the blood. As neutrophils, monocytes are also responsible for phagocytosis of foreign substances in the body. Monocytes can perform phagocytosis using intermediary (opsonising) proteins such as antibodies or complement that coat the pathogen, as well as by binding to the microbe directly via pattern-recognition receptors that recognize pathogens (324). Studies of phagocytosis and killing of *S. suis* by murine and human monocytes/macrophages showed similar results as those obtained with neutrophils. Studies carried out by different techniques such as viable count, bacterial staining or flow cytometry showed that virulent strains of *S. suis* are not (or almost never) phagocyted by porcine and human monocytes/macrophages in non opsonic conditions (48, 61, 222, 316, 338). These results corroborate that the capsule is the major resistance molecule against phagocytosis and killing by these leukocytes and that, as also occurs with neutrophils, specific-antibodies against this pathogen enhance the phagocytosis of *S. suis*. However, the increased of phagocytosis of *S. suis* by these leukocytes in presence of complement is controversial. Some studies have shown that complement alone does not help leukocytes to phagocyte *S. suis* (316, 346) but that it is important in the phagocytosis of the non-encapsulated mutant (222). However, on the other hand, it has been demonstrated that deactivated serum significatively increase the phagocytosis of *S. suis* by porcine and mice phagocytes (48, 61) which means that sera components other than complement are important for the phagocytosis of *S. suis* by the host immune system.

1.7.3. Septicemia

The pathogenesis of *S. suis* infection is poorly understood (128). There is evidence, however, that the immune system may play an important role in both the development of and protection against disease. Paradoxically, the failure to control this inflammatory cascade may be at the origin of clinical manifestations. Several inflammatory and infectious diseases are associated with the overproduction of proinflammatory cytokines and chemokines, and the recruitment and activation of different leukocyte populations are a hallmark of acute inflammation (322). These cytokines are believed to mediate responses associated with clinical deterioration, multiorgan

system failure and death from septic shock (385). It has been reported that *S. suis* serotype 2 is able to interact with leucocytes of porcine origin in a whole-blood system, inducing the upregulation of the proinflammatory mediators TNF-α, IL-1β, IL-6, IL-8 and MCP-1 (337). In addition, *S. suis* also provokes the release of TNF-α, and IL-6 by murine macrophages (335) and TNF-α, IL-6, IL-1, IL-8 and MCP-1 by human monocytes (336). The activation of these cytokines is CD14 and TLR2 dependent and the major *S. suis* component responsible of the production of these cytokines is the cell wall (132, 337). Moreover, it has also been described that suilysin also promotes the production of TNF-α by human monocytes and IL-6 by alveolar macrophages and porcine monocytes (221). The TNF-α, IL-1 and IL-8 cytokines are considered as essentials for the initiation of the inflammation but also for the leukocytes recruitment (128, 335). These feed-back positive signals produce an overproduction of cytokines that can conclude with the apparition of some important clinical signs in the host such as septicemia and finally death.

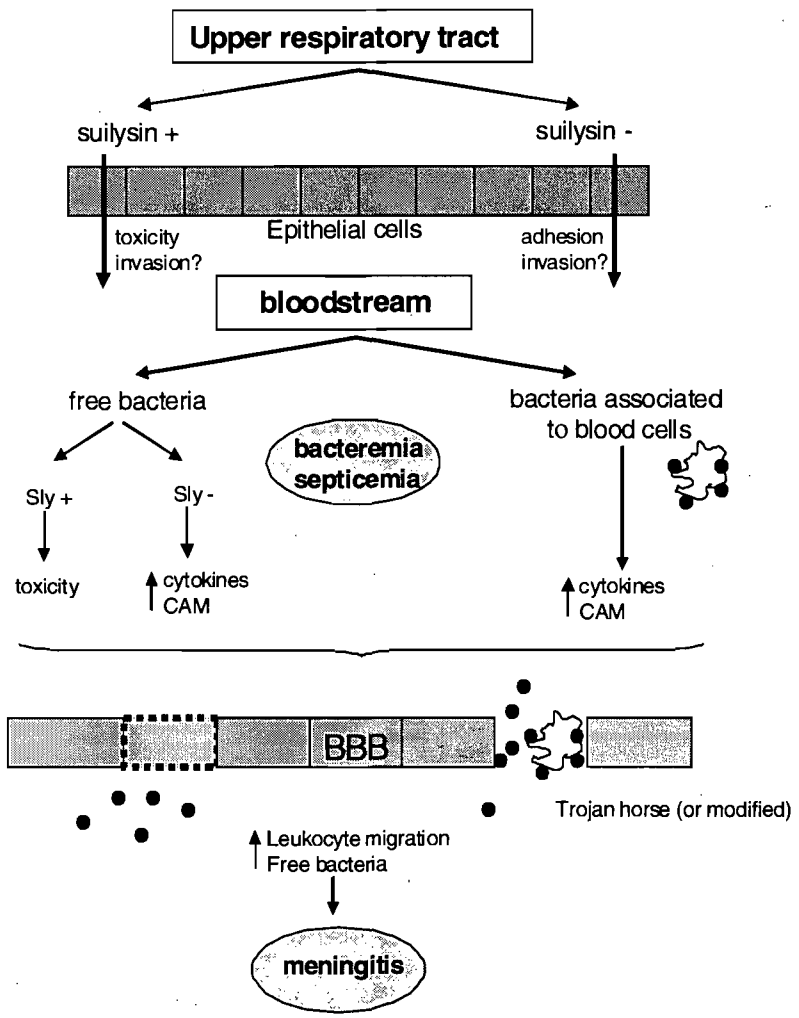

Fig. 1. Summary of the knowledge and proposed hypotheses for the different steps involved in the pathogenesis of meningitis of *S. suis*. *S. suis* interacts with the epithelial cell layers of the upper respiratory tract and penetrates to blood circulation. Sly+ strains may use toxicity and invasion to reach the bloodstream, while Sly− strains are still uncertain. *S. suis* could also be directly uptaken by blood cells and enter to the bloodstream within circulating cells. Bacteria could travel in bloodstream associated to these clood cells (bound and/or intracellular) or as free bacteria, resulting in bacteremia or septicemia. Free bacteria would enter the CNS after increasing BBB permeability, via direct cell toxicity (Sly+ strains), indirectly via local cytokine production or via other unknown(s) mechanism(s). Local cytokine production could also increase CAMs expression and leukocyte migration, that in turn "open the door" to free bacterial trafficking. On the other side, blood cells-associated bacteria would enter the CNS via the "Trojan horse" (bacteria inside cells) or "modified" Trojan horse (bacteria adhered to cells) mechanism favoured by activated phagocyte cytokine release. Figure adapted from (128)

1.7.4. Meningitis

In the event, for unknown reasons, that bacteria fail to cause acute fatal septicemia, *S. suis* is able to reach the central nervous system (CNS) through mechanisms that are only partially understood. To reach the CNS bacteria are facing the blood brain barrier (BBB). This consists of two levels: a barrier between blood and brain arises at the cerebral capillary endothelium and a barrier between blood and cerebrospinal fluid (CSF) at the choroid plexus epithelium. The BBB is characterised by restricted permeability on both sides of the layer due to the presence of tight junctions between endothelial cells of cerebral vessels and epithelial cells of the choroid plexus (372). Bacterial adherence to brain microvascular endothelial cells (BMEC) can consequently lead to invasion, toxicity and/or increase in permeability. Most meningeal bacteria such as group B streptococci, *S. pneumoniae* and *Escherichia coli* are able to adhere to and invade brain BMEC (259, 372). In the case of *S. suis* it has been reported that this bacterium is able to adhere to human and porcine BMEC (62, 378). In addition, a recent study using a mutant lacking sortaseA showed that this mutant had reduced capacity to adhere to and invade porcine brain microvascular endothelial cells compared to the wild-type strain indicating that adhesins of the MSCRAMMs family (see point 2.3.1) are, at least in part, responsible for this adhesions (380). Interestingly, invasion was proved only for the porcine cells, but not for the human cells, although both humans and pigs develop *S. suis* meningitis (62, 378). Moreover, it has been demonstrated that *S. suis* can resist up to seven hours inside porcine BMEC which could indicate a possible way to cross the BBB. In addition, toxins such as suilysin were shown to be toxic for cells forming BBB *in vitro* (62, 378). This cytotoxicity might produce functional alteration and increased permeability of BBB, which can help bacteria to penetrate across the endothelial barrier (320) but also the choroid plexus epithelial cells (364). In contrast, suilysin negative strains might rely on other mechanisms than toxicity to get access to the CNS (128). It has been hypothesised that after adherence to BMEC or leukocytes, *S. suis* might stimulate production of arachidonic acid, PGE_2, MMP9 and proinflammatory cytokines resulting in alteration of BBB permeability (87, 175, 176, 337). The local production of proinflammatory cytokines by microglial cells, endothelial cells, and migrating leukocytes upon contact with bacteria is currently regarded as the initial step for the development of meningitis. Several cytokines and chemokines (especially, TNF-α, IL-1, IL-8) are known to up-regulate the expression of cell adhesion molecules (CAMs), such as selectins and integrins, which allow transmigration of leukocytes through the barriers (128). The observed cytokine-inducing activity of *S. suis* may have significant biological relevance since it has been demonstrated that these cytokines can be generated in the blood. For example, in the monocyte-associated theory (Trojan horse or "modified"), cytokine release by activated phagocytes after interaction with *S. suis* might, in turn, activate endothelial cells and increase both cell and bound (or intracellular) bacterial trafficking. On the other side, if the free circulating bacterial theory is considered, *S. suis* could

directly, or indirectly (via local cytokine release) stimulate the expression of CAMs and non-infected leukocyte transmigration across the barriers. In this case, migrating leukocytes "open the door" for bacterial trafficking to different organs.

Once inside the BBB, *S. suis* is be able to survive and multiply in the cerebrospinal fluid and then cause damage to the CNS including brain oedema, increased intracranial pressure, and cerebrovascular insults provoking the meningitis.

1.8. Vaccines against *S. suis*

As explained before, *S. suis* infections can be treated by antibiotics. However, the antibiotic resistance found in some of the strains and the fact that it is essential to start the treatment at the beginning of the infection to avoid bacterial transmission between pigs in the herds, show the importance of the development of new strategies. In the last years, different types of vaccines have been developed or are presently under investigation. However, up to now, the results obtained are not very encouraging.

The idea of creating a vaccine against *S. suis* infections appears when Elliot *et al.* showed that the transfer of serum from convalescent piglets resulted in complete protection against subsequent *S. suis* infection (92). These results indicate that some *S. suis* antigens are able to produce a specific immune response able to prevent subsequent *S. suis* infections. Some years later, Holt and colleagues (159, 160) identified, by using sera raised against different fractions of *S. suis* type 2, six major surface antigens of 44, 78, 86, 94, 130 and 136 kDa which were not recognized by serum from unprotected animals. They demonstrated that sera against the 94 kDa protein protected mice against challenge with a live, virulent strain of *S. suis* type 2. However, that study also showed that the serum raised against the 78 kDa protein inhibited the protective effect of the 94 kDa antiserum. Although the 94 kDa protein conferred protection in mice, its use as a possible subunit vaccine to control *S. suis* type 2 infection in pigs is not possible because it would require elimination of the 78 kDa antigen. Moreover, it is not known whether this protein is produced by other serotypes and will therefore be cross-protective, or whether it is serotype specific. Antibodies against the other surface proteins identified in that study were variably opsonic, but none was protective.

Quessy and colleagues also reported a protective effect against heterologous challenge to mice with virulent strains of *S. suis* type 2 of a 110 kDa protein purified from a culture supernatant of *S. suis* type 2 (295). However, this protein was never characterized sufficiently to determine its usefulness as a possible subunit vaccine.

In another effort to search for a *S. suis* protein(s) that can be used as a vaccine candidate, Okwumabua *et al.* constructed and screened a gene library with a polyclonal antibody raised against the whole-cell protein of *S. suis* type 2 (264). A clone that reacted with the antibody was identified

and characterized. The amino acid sequence of this protein shared homology with sequences of unknown function from other streptococci. They demonstrated that pigs immunized with this recombinant 38-kDa protein mounted antibody responses to the protein and were completely protected against challenge with a strain of a homologous serotype, a wild-type virulent strain of *S. suis* type 2, suggesting that it may be a good candidate for the development of a vaccine against *S. suis* infection. However, it is has never been demonstrated that this protein is protective against other *S. suis* serotypes.

On the other hand, vaccination strategies using known potential virulence factors such as purified suilysin (168) or MRP and EF (397) from *S. suis* serotype 2 have been shown to protect pigs from homologous and heterologous serotype 2 strains. However, and as explained before, a substantial number of virulent strains in some geographic regions do not express these proteins and, this feature impede the use of these proteins as an universal vaccine.

Another *S. suis* critical virulence factor tested as vaccine was the CPS. However, its poor immunogenicity also impedes the use of this molecule as a vaccine (93). *S. suis* capsule biosynthesis has not yet been fully elucidated. In two subsequent studies, the *S. suis* serotype 2 *cps2* was identified as an important gene for capsule biosynthesis and non-encapsulated mutants were generated by disruption of this gene (346). However, a recent study showed that inactivation of the *aro* operon in *S. suis* serotype 2 also resulted in an unencapsulated phenotype (106). As one of the several approaches that may be used to elicit protective immunity against this bacterium is vaccination with a live, attenuated strain of *S. suis*, the use of these mutants was tested. Results obtained with both mutants were variable. The study using the unencapsulated Δ*cps2* mutant as live vaccine showed that this mutant failed to provide protection to pigs against the homologous serotype 2 parent strain (346) and that, as consequence, this strain is not usefull as a vaccine. In contrast, vaccination with the unencapsulated *aro* mutant provided a relatively good protection level against a challenge with the homologous serotype 2 parent strain. However, this protection was not 100% effective and, in addition, it has not been tested against other *S. suis* serotypes, which impede, for the moment, its use as a effective vaccine (106).

Repeated injections with other virulent, avirulent, or formalin-killed virulent *S. suis* type 2 also gave variable results. Live avirulent strains protected against serotype 2 challenge in pigs (157, 158, 296) but did not eliminate already established organisms or prevent the establishment of carriers (158). Single immunization with formalin-killed virulent type 2 organisms resulted in a good opsonizing response, although this did not always correlate with protection (157). In addition, passive protection studies in mice using the same formalin-killed strain showed protection with serum from hyper-immunized pigs but none with serum from piglets (42). Kebede and colleagues also tested the protective value of temperature-sensitive mutants of *S. suis* and only homologous protection was achieved (182). Finally, Foster and colleagues evaluated the use of a streptomycin-

dependent mutant of *S. suis* type 1/2 as a vaccine. Although homologous protection was achieved, the vaccine failed to protect completely against *S. suis* type 2 (112).

At present, the most used vaccines in the field are the inactivated commercial autogenous vaccines, but results have been inconsistent (142, 203). Simonson *et al.* described that the use of commercial bacterins (STREP BACT-TM) are highly effective to confer passive and active immunisation against serotype 1 and 2 of *S. suis*. However, this protection is limited since other serotypes can also produce infections (41).

Up to now, an effective vaccine able to protect pigs or humans against all virulent serotypes of *S. suis* does not exist. However, several research groups are now working hard to find new antigenic *S. suis* surface antigens, present in all the *S. suis* serotypes, that could finally confer a successful protection against this pathogen.

As explained before, bacterial virulence is determined by a wide variety of factors that influence bacterial attachment, penetration into tissue, and the escape from host. Up to now, different bacterial ECM-binding proteins have been suggested as potential vaccine targets for preventing bacterial infections because antibodies against these surface proteins are believed to have the dual activity of both adherence blocking and opsonic function (109).

2. Bacterial adhesion to Extracellular Matrix:

2.1. Extracellular matrix

2. 1. 1 General composition

A major portion of tissues is extracellular space that is filled by a network of macromolecules secreted by the cellular components within the tissue. ECM is present to some degree in all tissues, even blood where the plasma might be considered the matrix, but is a functional determinant in connective tissues (9). The matrix is mainly composed of structural and adhesive proteins.

2.1.1.1. Structural ECM components

The principal structural components of the eukaryotic ECM are collagens that form different types of interstitial or basement membrane networks, including fibril-forming collagens (collagen types I, II, and III) and the two-dimensional collagen type IV network. For further organization of the ECM, additional collagenous glycoproteins, proteoglycans, hyaluronan, and many other components, such as growth factors and proteases, become associated with interstitial or basement-membrane ECM, giving rise to their specialized structure and function at different locations in the body (9).

The main portion of interstitial ECM is produced and deposited by different connective tissues cells and determines the specific character of each tissue or organ (9). Consequently, the tropism of pathogenic microorganisms is determined by the local composition of this complex network of host-derived factors.

2.1.1.1.1. Collagen

Collagen is found in all of the connective tissues, such as dermis, bones, tendons, and ligaments, and also provides the structural integrity of all of our internal organs (53). Therefore, because of its wide distribution throughout our bodies, it represents one of the most abundant naturally occurring proteins (376).

There are close to 20 different types of collagen (246). Each one of these collagens is encoded by a specific gene. The predominant form is Type I collagen. This fibrillar form of collagen represents over 90 percent of our total collagen and is composed of three very long protein chains. Each chain is referred to as an "Alpha" chain. The amino acid sequence of each chain often follows the pattern Gly-X-Pro or Gly-X-Hydroxyproline, where X may be any amino acid residues. In collagen, Gly is required at every third position because the assembly of the triple helix puts this residue at the interior (axis) of the helix, where there is no space for a larger side group than glycine's single hydrogen atom. For the same reason, the rings of the Pro and Hyp must point outward. These two amino acids thermally stabilize the triple helix. This amino acid sequence

permits that the three Alpha chains are wrapped around each other to form the final triple helical structure called a collagen monomer. This configuration imparts tremendous strength to the protein.

Basically all of the collagen types (131) share this triple-helical molecular structure. However, the various other types of collagens have slightly different amino acid compositions and provide other specific functions in our bodies (131). Type II collagen is the form that is found exclusively in cartilaginous tissues. It is usually associated with proteoglycans or "ground substance" and therefore functions as a shock absorber in our joints and vertebrae. Type III collagen is also found in our skin as well as in blood vessels and internal organs. In the adult, the skin contains about 80-percent Type I and 20-percent Type III collagen. In newborns, the Type III content is greater than that found in the adult. It is thought that the supple nature of the newborn skin as well as the flexibility of blood vessels is due in part to the presence of Type III collagen. During the initial period of wound healing, there is an increased expression of Type III collagen (70). Type IV collagen is found in basement membranes and functions as a filtration system. Because of the complex interactions between the Type IV collagen and the noncollagenous components of the basement membrane, a meshwork is formed that filters cells as well as molecules and light. Finally, Type V collagen is found in essentially all tissues and is associated with Types I and III. In addition, it is often found around the perimeter of many cells and functions as a cytoskeleton.

The biosynthetic pathway responsible for collagen production is very complex (291). Each specific collagen type is encoded by a specific gene. The messenger RNA for each collagen type is transcribed from the gene and undergoes many processing steps to produce a final code for the specific collagen type. A precursor form of collagen called procollagen is produced initially (31). Procollagen contains extension proteins on each end called amino and carboxy procollagen extension propeptides. These nonhelical portions of the procollagen molecule make it very soluble and therefore easy to move within the cell as it undergoes further modifications. The first modification to take place is the very critical step of hydroxylation of selected proline and lysine amino acids. Specific enzymes called hydroxylases are responsible for these important reactions forming hydroxyproline and hydroxylysine (254). After that, some of the newly formed hydroxylysine amino acids are then glycosylated by the addition of sugars, such as galactose and glucose (16) by the galactosyl or glucosyl transferases enzymes. This glycosylation step imparts unique chemical and structural characteristics to the newly formed collagen molecule that influence fibril size (187).

As explained above, when the procollagen peptides are intact, the molecule is about 1,000 times more soluble than it is at a latter stage when the extension peptides are removed (292). This high degree of solubility allows the procollagen molecule to be transported, by means of specialized structures called microtubules, within the cell and finally secreted into the extracellular spaces (86).

Once secreted, specialized enzymes called procollagen proteinases remove both of the extension peptides from the ends of the molecule and give the typical insolubility to the collagen (202). It is also in the extracellular spaces where the rest of post-translational modifications take place. The triple helical collagen molecules line up and begin to form fibrils to finally form the collagen fibers. This step is called crosslink formation and is promoted by another specialized enzyme called lysyl oxidase (27). This last reaction places stable crosslinks within (intramolecular crosslinks) and between the molecules (intermolecular crosslinks) giving the tremendous strength to the collagen fibers present in the tissues.

2.1.1.1.2. Laminin

In addition to the network-forming collagen IV, the mature basement membranes also contain other abundant structural components. Laminin is the most abundant noncollagenous protein in the basement membrane and is responsible for stability (407).

Up to now, eleven different genes encode the eleven chains of the laminin protein family (α1–5, β1–3, and γ1–3). This α-, ß-, or γ-chain denomination is based on sequence identity and protein domain organization. There is some evidence that a fourth β-chain could exist, but its existence is currently unclear (210). The α-chain averages 400 kDa in size and 160 nm in length. The β-chain averages 215 kDa in size and 60 nm in length. Finally, the γ-chain averages 205 kDa in size and 40 nm in length respectively.

Structurally, laminin are formed by heterotrimeric chains that resemble a three-pronged fork (Fig 2). The C-termini of the α-, β-, and γ-chains form the "handle" of the laminin fork. The three "prongs" are the short arms of laminin that emerge from the coiled-coil domain (210).

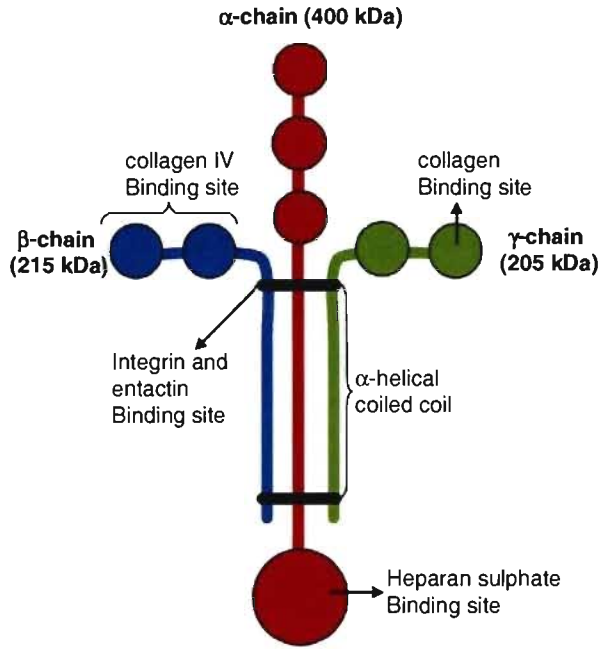

Fig 2: Diagram of the Laminin fork structure composed of three different chains: α, β and γ. Figure adapted from (371)

Laminin plays an essential role in basement membrane formation due to multiple interactions with itself and other components. Laminins contain binding sites for other cell components such as integrins, heparin sulphate, proteoglycans, dystroglycan, nidogen, and lutheran (Fig 2). These adhesions permit that laminin influence multiple functions of adjacent cells including adhesion, proliferation and differentiation (247, 367).

Laminin assembly is a complex process that up to now is not completely understood. It seems that once in the Golgi apparatus, ionic interactions favor βγdimer formation, which is then stabilized and secreted upon incorporation of the α-chain (260, 371). The point of intersection of the three chains is stabilised by disulfide bridges (280). In addition, the rod or "handle" of the fork is stabilized by a heptad repeats. These heptad repeats allow for hydrophilic, charged, and hydrophobic stabilization of the trimer. (371). Much like type IV collagen, laminin seems to be able to self-assemble in basement membranes into a honeycomb-like polymer. This self-assembly of the heterotrimeric laminins into oligomers is a process dependent of calcium and is attributed to the globular domain of each chain (Fig 2), although the mechanism remains elusive (281, 371, 408).

2.1.1.1.3. Elastin

Many vertebrate tissues, such as skin, blood vessels, and lungs, need to be both strong and elastic in order to function. A network of elastic fibers in the ECM of these tissues gives them the required resilience so that they can recoil after transient stretch (276). Long, inelastic collagen fibrils are interwoven with the elastic fibers to limit the extent of stretching and prevent the tissue from tearing.

The main component of elastic fibers is elastin, a highly hydrophobic protein (about 750 amino acids long), which, like collagen, is unusually rich in proline and glycine but, unlike collagen, is not glycosylated and contains some hydroxyproline but no hydroxylysine (319).

The elastin protein is composed largely of two types of short segments that alternate along the polypeptide chain: i) the hydrophobic segments, which are responsible for the elastic properties of the molecule, and ii) the alanine- and lysine-rich α-helical segments, which form cross-links between adjacent molecules (319). Each segment is encoded by a separate exon. There is still controversy, however, concerning the conformation of elastin molecules in elastic fibers and how the structure of these fibers accounts for their rubber-like properties. The most accepted theory is that elastin polypeptide chain adopts a loose "random coil" conformation, and it is the random coil structure of the component molecules cross-linked into the elastic fiber network that allows the network to stretch and recoil like a rubber band (319).

Elastin synthesis goes through several steps similar to those observed in the collagen formation: gene transcription, alternative splicing of pre-mRNA, mRNA translation, hydroxylation of some proline residues of the tropoelastin, secretion of tropoelastin molecules in the extracellular

space, synthesis of cross-links with lysinonorleucine, desmosine and isodesmosine, that gives insolubility and elasticity of elastin, and finally, deposition of this elastin on the microfibrillar scaffold (166).

As explained above, elastic fibers are not composed solely of elastin. The elastin core is covered with a sheath of microfibrils, each of which has a diameter of about 10 nm (315). Microfibrils are composed of a number of distinct glycoproteins, including the large glycoprotein fibrillin, which binds to elastin and is essential for the integrity of elastic fibers. Microfibrils are thought to be important in the assembly of elastic fibers. They appear before elastin in developing tissues and seem to form a scaffold on which the secreted elastin molecules are deposited. As the elastin is deposited, the microfibrils become displaced to the periphery of the growing fiber stabilizing it (315).

2.1.1.2 Adhesive glycoproteins

Upon vessel wall injury, particularly at sites of wound healing, initial adhesion of platelets to the exposed subendothelium and subsequent platelet aggregation are dependent on adhesive glycoproteins present in the subendothelial cell matrix as well as those stored inside platelets and secreted during this initial phase of hemostatic plug formation (9). In addition to their strong attachment-promoting activity, residing predominantly in the Arg-Gly-Asp (RGD)-containing epitope and being the predominant recognition site for integrins, these multifunctional proteins are of major importance in the initial adherence phase of pathogens (9).

2.1.1.2.1. Fibronectin

Fibronectin is one of the most important ECM proteins because it has numerous functions that ensure the normal functioning of life. One of its more notable functions is its role as a 'guide' in cellular migration pathways in mammalian development, particularly the Neural Crest (ectoderm cells that will develop into skin pigment cells as well as some bones of the skull). Moreover, fibronectin also helps maintain the shape of cells by lining up and organizing intracellular cytoskeleton by means of receptors. In addition, it helps stabilize the attachment of ECM to cells by acting as binding sites for cell surface receptors. More generally though, fibronectin helps create a cross-linked network within the ECM by having binding sites for other ECM components (355).

Fibronectin can be found in two different forms (227). The blood plasma fibronectin, which is a soluble form, composed of two 250 kDa subunits joined together by disulfide bonds and the cellular fibronectin. The latter insoluble form, that was formerly called cold-insoluble globulin, is synthesized by the cells that form the ECM and is formed by a large complex of cross-linked subunits. Each fibronectin subunit is folded into a series of functionally distinct domains separated by regions of flexible polypeptide chain (Fig 3). The domains in turn consist of smaller modules, each of which are serially repeated and usually encoded by a separate exon, suggesting that the fibronectin gene, like the collagen genes, evolved by multiple exon duplications (227). All isoforms

of fibronectin are encoded by a single large gene that contains about 50 exons of similar size. Transcription produces a single large RNA molecule that can be alternatively spliced to produce the various isoforms of fibronectin (227).

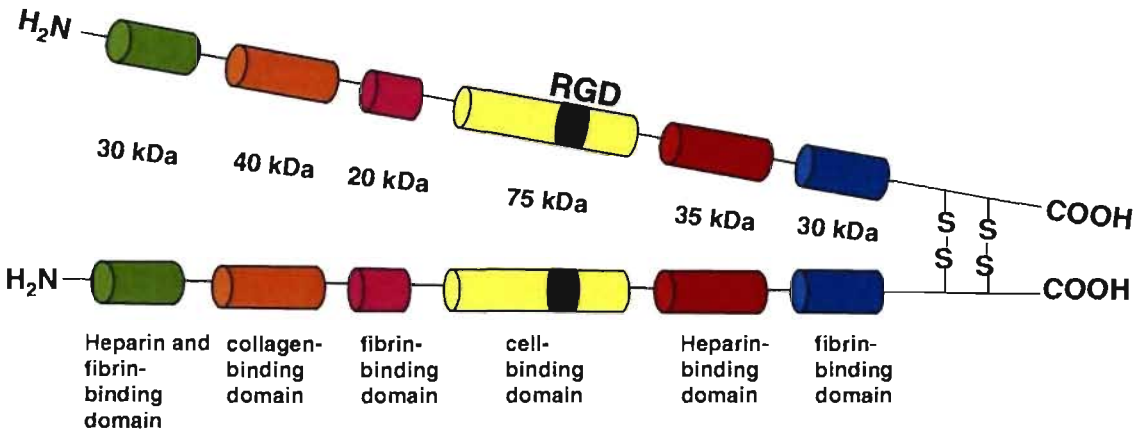

Fig 3: Diagram of the fibronectin structure. Figure adapted from www.bbioo.com

When fibronectin is treated with a low concentration of a proteolytic enzyme, the polypeptide chain is cut in the connecting regions between the domains, leaving the domains themselves intact. One can then show that one of its domains binds to collagen, another to heparin, and the last one to specific receptors on the surface of various types of cells (Fig 3) (227). Synthetic peptides corresponding to different segments of the cell-binding domain have been used to identify a specific tripeptide sequence (Arg-Gly-Asp, or RGD) (227). This RGD sequence is not confined to fibronectin. It is also found in a number of extracellular proteins, including, for example, the blood-clotting factor fibrinogen. RGD sequences are recognized by several members of the integrin family of cell-surface matrix receptors. Each integrin, however, specifically recognizes its own small set of matrix molecules, indicating that tight binding requires more than just the RGD sequence (360).

Unlike fibrillar collagen molecules, which can be made to self-assemble into fibrils in a test tube, fibronectin molecules assemble into fibrils only on the surface of certain cells (285). This is because additional proteins are needed for fibril formation, especially fibronectin-binding integrins (227). The fibronectin fibrils on the cell surface are highly stretched and under tension. The tension is exerted by the cell and is essential for fibril formation (227). The fibronectin fibrils are usually aligned with adjacent intracellular actin stress fibers. In fact, intracellular actin filaments promote the assembly of secreted fibronectin molecules into fibrils and influence fibril orientation. The interactions between extracellular fibronectin fibrils and intracellular actin filaments are mediated mainly by integrin transmembrane adhesion proteins. The contractile actin and myosin cytoskeleton thereby pulls on the fibronectin matrix to generate tension. As a result, the fibronectin fibrils are stretched, exposing a cryptic (hidden) binding site in the fibronectin molecules that allows them to

bind directly to one another. In addition, the stretching exposes more binding sites for integrins. In this way, the actin cytoskeleton promotes fibronectin polymerization and matrix assembly (227).

2.1.1.2.2. Vitronectin

Vitronectin is present in normal plasma at concentrations of 200 to 400 µg/ml and constitutes 0.2–0.5% of total plasma protein (28). Previous data suggests that the liver is a major site of vitronectin biosynthesis (339). A second circulatory pool of vitronectin is contained within platelets. This rapidly releasable intracellular form accounts for only 0.8% of the circulating vitronectin pool (275). A high level of vitronectin mRNA is present in the liver. However, many other organs including brain, heart, skeletal muscle, lung, uterus, testis and thymus were also found to contain vitronectin mRNA (339).

The molecular weight of human vitronectin is 75 kDa. It circulates as a single-chain (75 kDa) and as a two-chain (10 and 65 kDa) form under reducing conditions. Under non-reducing conditions, the N-terminal 65 kDa and C-terminal 10 kDa fragments are linked by a single disulfide bond (369) (fig 4).

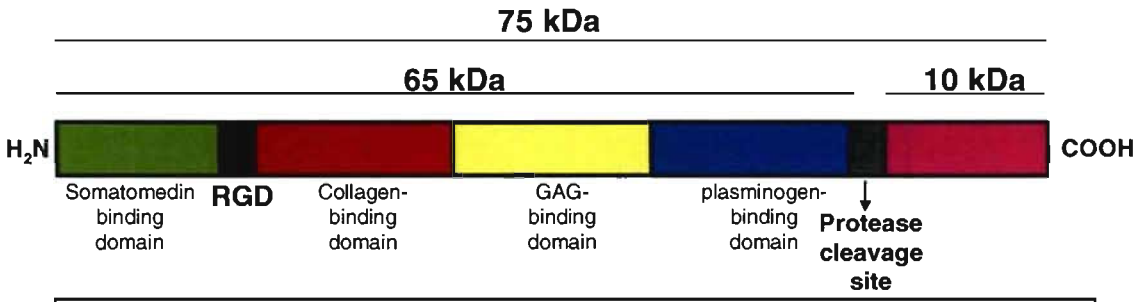

Fig 4: Diagram of the vitronectin structure. Figure adapted from www.haemtech.com

Vitronectin contains three glycosylation sites that contribute approximately 30% of its molecular mass. The complete open reading frame of vitronectin encodes for 459 amino acids preceded by a signal peptide of 19 amino acids in length (328). The vitronectin gene consists of eight exons and seven introns, resulting in a 1.7 kb transcript with no indication for alternative splicing (289).

Vitronectin is a conformationally labile molecule (fig 4). This lability likely plays a large role in regulating its function. Formation of complexes with plasminogen activator inhibitor-1 (PAI-1), thrombin–antithrombin III, or complement C5b–C9 induces conformational changes within vitronectin exposing its heparin binding domain. Interestingly, conformational changes are not limited to the heparin binding domain and also occur in the N-terminal half of the molecule including the somatomedin B domain (289). This region of the vitronectin molecule contains a high

affinity binding domain for PAI-1 (340) and an Arg-Gly-Asp (RGD) amino acid sequence. A highly acidic region (aa 53–64) containing sulfation sites (tyrosine residue 56 and 59), a putative crosslinking site (amino acid 93), and a collagen binding domain occurs adjacent to the RGD sequence (165). Due to its RGD sequence, vitronectin plays a key role in the attachment of cells to their surrounding matrix. Vitronectin is anchored to the extracellular matrix by its collagen binding and glycosaminoglycan (GAG) binding domains. The carboxy-terminal region of vitronectin (aa 332–348) accommodates its plasminogen binding domain (191). A cluster of basic amino acids containing two consensus heparin binding sites occurs downstream of this region. This site is particularly exposed in vitronectin multimers engaged in specific binding interaction with collagen (type 1), osteonectin, and PAI-1 (191).

Vitronectin has been implicated as a regulator of many diverse physiological processes including coagulation, fibrinolysis, pericellular proteolysis, complement dependent immune response, cell attachment and spreading (341). Vitronectin is involved in fibrinolysis due to its ability to stabilize the active conformation of PAI-1 and bind the urokinase receptor. Vitronectin is also involved in immune defense via interaction with the terminal complex of complement and in haemostasis via heparin binding which neutralize antithrombin III inhibition of thrombin and factor Xa (289).

2.1.1.2.3. Fibrinogen and fibrin

Fibrinogen is a soluble glycoprotein found in the plasma, with a molecular weight of 340 kDa (88). Fibrinogen has a biological half-life of about 100 h and is synthesized predominantly in the liver (141). As a clotting factor, fibrinogen is an essential component of the blood coagulation system, being the precursor of fibrin (179). However, at the 'usual' plasma levels of 1.5 to 4.5 g/l, its concentration far exceeds the minimum concentration of 0.5–1 g/l necessary for haemostasis (179).

The fibrinogen molecule is a hexamer consisting of two identical sets of three polypeptide chains (α, β, and γ) held together by disulfide bonds (146) (Fig 5). Synthesis of the protein in hepatocytes is under the control of 3 genes (one for each chain) located within 50 kilobases (kb) on chromosome 4 (180).

Fibrinogen resembles fibronectin in that fibrinogen also contains many important functional binding regions, including heparin binding domains on the N-terminal β chain (404), platelet recognition sites (143) and ICAM-1 binding sites (18). Other important sites on fibrinogen include the Arg-Gly-Asp (RGD) sites (115) which, as explained above, are necessary for integrin ligation permitting adhesion of endothelial cells to provisional matrix through specific integrins.

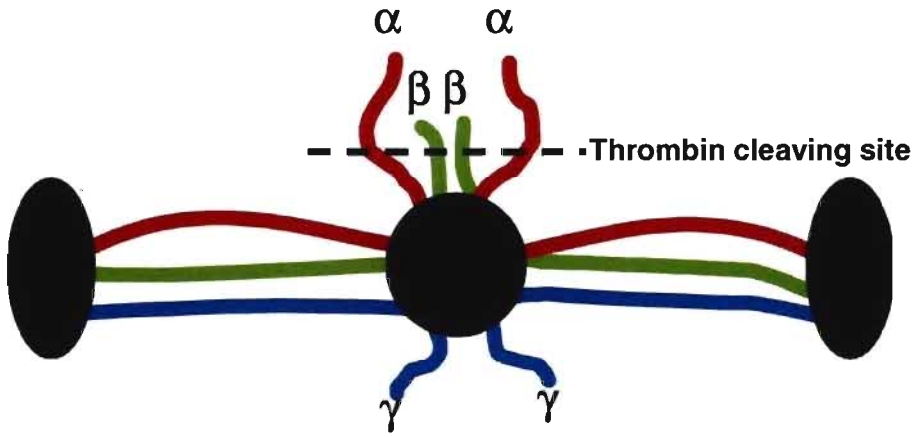

Fig 5: Diagram of the fibrinogen/fibrin structure. Figure adapted from (293)

Processes in the coagulation cascade (Fig 6) activate the zymogen prothrombin to the serine protease thrombin, which is responsible for converting fibrinogen into fibrin (134). Thrombin causes a limited proteolysis of the fibrinogen molecule, during which fibrinopeptides A and B are released from the N-terminal regions of the α and β chains, respectively (Fig 5). These fibrinopeptides, which have been sequenced in many species, may have a physiologic role as vasoconstrictors and may aid in local haemostasis during blood clotting. In addition, fibrinogen fragments also function as chemotactic factors for inflammatory cell infiltration (390). Thrombin is also responsible for the activation of the fibrin-stabilizing factor, which in its activated form is a transpeptidase catalyzing the formation of epsilon-(gamma-glutamyl)-lysine crosslinks in fibrin to form a clot (325). Together with vitronectin and other adhesion proteins, the fibrin clot constitutes the majority of the initial provisional ECM network for sealing a wound site, thereby protecting this area against infiltration by opportunistic, infectious microorganisms (204).

On the other hand, fibrinolysis is the process wherein a fibrin clot, the product of coagulation, is broken down (238). Its main enzyme plasmin cuts the fibrin mesh at various places, leading to the production of circulating fragments that are cleared by other proteases or by the kidney and liver (56). Plasmin is produced in an inactive form, plasminogen, in the liver. Although plasminogen cannot cleave fibrin, it still has an affinity for it, and is incorporated into the clot when it is formed (56). Plasminogen contains secondary structure motifs known as kringles, which bind specifically to lysine and arginine residues on fibrin(ogen). When converted from plasminogen into plasmin, it functions as a serine protease, cutting C-terminal to these lysine and arginine residues (56). Tissue plasminogen activator (t-PA) and urokinase are the agents that convert plasminogen to the active plasmin, thus allowing fibrinolysis to occur (238). t-PA is released into the blood very slowly by the damaged endothelium of the blood vessels, such that, after several days (when the bleeding has stopped), the clot is broken down. This occurs because plasminogen becomes entrapped within the clot when it is formed; as it is slowly activated, it breaks down the fibrin mesh.

t-PA and urokinase are themselves inhibited by plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2 (PAI-1 and PAI-2) (238). In contrast, plasmin further stimulates plasmin generation by producing more active forms of both tPA and urokinase (238). On the other hand, alpha 2-antiplasmin and alpha 2-macroglobulin inactivate plasmin. Plasmin activity is also reduced by thrombin-activable fibrinolysis inhibitor, which modifies fibrin to make a less potent cofactor for the tPA-mediated plasminogen activation (238).

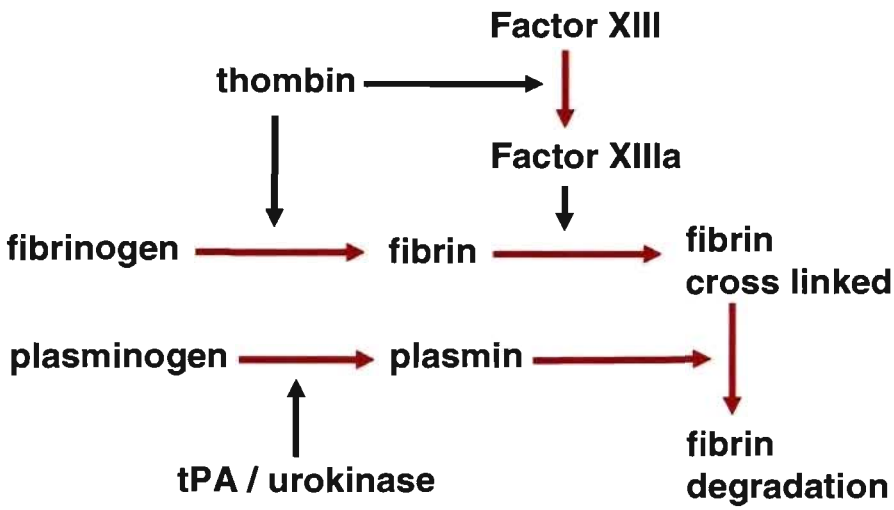

Fig 6: Coagulation cascade of proteins. Figure adapted from (293)

2.1.1.2.4. Thrombospondin

Thrombospondin is a high-molecular weight, heparin-binding glycoprotein that was initially termed "thrombin-sensitive protein" based upon its release by thrombin-activated platelets (25, 26). Thrombospondin is one of the most abundant proteins in the platelet α-granule (77, 359). Structurally, thrombospondin is a 450,000 Da molecular weight glycoprotein (208) consisting of three, identical, disulfide-linked polypeptide chains (206). Thrombospondin is not an exclusive product of platelets and megakaryoctes. It can be also synthesised by endothelial cells (252), fibroblasts (170), monocytes and macrophages (169), and osteoblasts (308).

Over the last decade it has become clear that platelet-derived thrombospondin, or thrombospondin 1 (TSP1) as it is now known, is a member of a family of at least five proteins, every one of which exhibits a distinct tissue distribution. It is sometimes referred to as the subgroup A thrombospondins which also contains thrombospondin 2 (TSP2). Subgroup B thrombospondins comprise the other members of the family: thrombospondins 3 to 5 (TSP3–5) which are homopentameric with smaller subunit masses than TSP1 or TSP2 (44).

All TSPs (Fig 7) have in common a region of about 650 amino acids at the carboxy-terminal end of their subunits. These amino acids are organised into domains or repeats that bind calcium and a range of extracellular molecules, and possess some cell attachment properties (3). The amino-terminal domain binds heparin and is thus also called the heparin binding domain (96). Following this domain, TPS has a collagen-binding domain that permit adhesion of this protein to the ECM and a set of domains, known as type 1 or properdin domains, that are anti-angiogenic (327). Subgroup B thrombospondins lack type 1 domains, do not inhibit angiogenesis and have variable amino-terminal domains. Type 2 domain, also termed epidermal growth factor- (EGF-) like repeats, is found after the type 1 domain and is variable in number. At the carboxy-terminal domain, TSPs contain the RGD motif important for the binding of this protein to the cell.

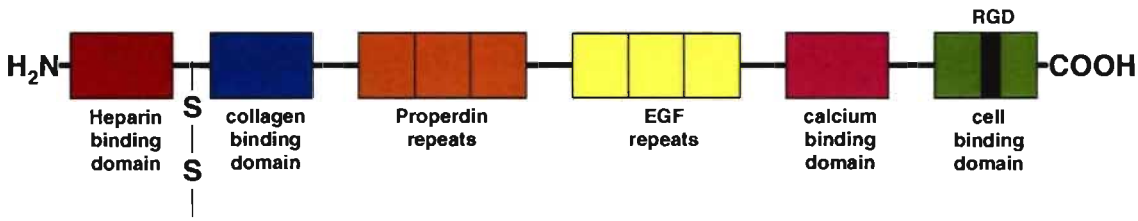

Fig 7: Diagram of the thrombospondin structure. Figure adapted from www.haemtech.com

Differences in structure and in tissue distribution between TSPs indicate that their physiological roles are likely to differ. Nevertheless, TSPs do have a number of analogous activities. For instance, both proteins bind calcium and heparin. However, these latter interactions represent only a fraction of their binding repertoire. Studies with platelet thrombospondin (TSP1) demonstrate that the protein interacts with components of platelets and of the clotting and fibrinolytic systems including fibrinogen, thrombin, plasminogen and osteonectin (67, 140, 206, 214). These findings raise the possibility that thrombospondin might promote platelet aggregation and clotting (207, 269). Another group of interactions between subgroup A thrombospondins and extracellular components are those involving structural and adhesive proteins. Mumby and colleagues reported that TSP1 could bind type V collagen, laminin and fibronectin. Subsequently, evidence emerged that TSP1 can also bind collagen types I–IV (253). Following studies concerning the molecular interactions of TSP1, Lahav and coworkers suggested that the protein plays a role in extracellular matrix (ECM) assembly (198).

Despite the complexity of TSPs interactions at the molecular level and their sometimes bewildering consequences on cellular behaviour, distinct multiple roles for the proteins in biological processes are starting to come to light. Thus, although the two proteins have major functions in development, it seems that it is in tissue formation during adult life where TSPs have a more overt impact (3). In normal adults TSPs appear to play a role in continued tissue turnover as evidenced by

their tightly controlled post-natal expression (44). For example, TSP1 is prominent in basement membranes, and at the base of epithelia in some adult human tissues (2, 67). It is conceivable that the role of the protein in these locations is related to collagen fibrillogenesis and/or cell differentiation (3). In addition, TSPs are also required for "normal" cutaneous wound healing. TSP1, derived from platelets and the monocytic cells of the inflammatory response, appears early in repair and is involved in coagulation and the provisional matrix, cell recruitment, apoptosis of damaged cells, protease modulation and TGFβ activation (7, 45). Conversely, TSP2 emanates from wound fibroblasts and so emerges later than TSP1 in repair. TSP2 seems to regulate angiogenesis and collagenous matrix assembly during healing (45, 197).

2.2. Bacterial adhesion to extracellular matrix protein (ECM)

An essential step in successful colonization and production of disease by microbial pathogens is their ability to adhere to host cell surfaces. Although different pathogens can adhere to nearly any site in the body, each pathogen usually colonizes one or a few particular sites in the host (391). The choice of host cell surface that a pathogen can adhere to is large. The mammalian cell surface contains many proteins, glycoproteins, glycolipids, and other carbohydrates that could potentially serve as a receptor for a bacterial adhesin. Additionally, the ECM provides a rich source of glycoproteins to which many pathogens bind (9).

As explained above, the ECM is the extracellular part of animal tissue that usually provides structural support to the cells. The ECM includes the interstitial matrix, present between various cells (in the intercellular spaces), and the basement membrane (356). Basement membranes are sheet-like depositions of ECM on which various cells rest. ECM is intimately linked and determines a variety of cellular systems, cellular shape, orientation, differentiation, and metabolism (9). Furthermore, the time-depending modification and rebuilding of ECM at different parts in the body is essential for inflammation and wound healing. At these sites, the provisional ECM may provide bacterial entry and colonization, whereas intact tissues or epithelia are mostly protected against bacterial infection by a variety of adhesive and mucoidal components (391). However, some pathogens can use bacterial toxins to cause tissue damage by destruction of host cells, an as consequence, exposure of the ECM. Once exposed, bacteria can bind these ECM proteins via its adhesins (391).

Bacterial adhesins for ECM proteins are usually proteins, either located at the tip of a scaffold-like structure on the bacterial surface (called fimbria) or anchored in the bacterial membrane but surface exposed (nonfimbrial or afimbrial adhesins) (391). It is becoming apparent that the molecular machinery needed to build pili or to transport an afimbrial adhesin to the bacterial surface is often conserved. Despite this conservation, the receptor specificity of the

adhesin is dictated by a portion of the adhesin exposed at the pilus tip or on the bacterial surface (391).

The bacterial binding to ECM proteins can have many consequences. On one hand these adhesions can mask the microbial surface and thereby interfere with antigen presentation and provide an overall immune evasion strategy. On the other, it can serve as a bridge between the bacterium and host cell surface when ECM proteins bind to their natural receptor on host cell surfaces (391) (Fig. 8). One of these natural receptors is integrins. Adhesion of pathogen to these integrins via ECM proteins is usually the first step in penetrating into the body through activation of host cell cytoskeleton (Fig. 8). This activation permits bacterial invasion to host cells and can be used by pathogens to cross some host barriers (391).

2.3. Bacterial fibronectin-binding proteins

Pathogenic bacteria often use adherence to and invasion of eukaryotic cells for colonization, evasion of immune defence and survival, as well as to cause disease in the host (172). Many Gram-positive bacteria express specific cell-surface components called adhesins that mediate their adherence to host cells, thereby facilitating not only colonization but also invasion (391). Most of these adhesins and invasins function by binding to various components of the extracellular matrix (ECM), particularly fibronectin (172). Many Gram-positive bacteria that interact with fibronectin have been identified (330). As explained before, fibronectin is a multi-functional protein that is abundant in the circulation and at various extracellular sites (227). As it can bind to both host cells and bacteria, it is considered an essential binding molecule for bacterial adherence. Moreover, via its interaction with integrins, fibronectin can also play a role in triggering the signal transduction events that lead to bacterial invasion of eukaryotic cells (330).

Up to now, three different types of fibronectin-binding proteins have been described for Gram-positive bacteria (65): i) microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), ii) choline-binding proteins and finally iii) anchorless-proteins.

Several Gram-positive bacteria, including streptococci and staphylococci, specifically bind to fibronectin using several different adhesins with highly homologous recognition motifs. These adhesins are anchored on the bacterial surface via an LPXTG motif and known as microbial surface components recognizing adhesive matrix molecules or MSCRAMMs (177).

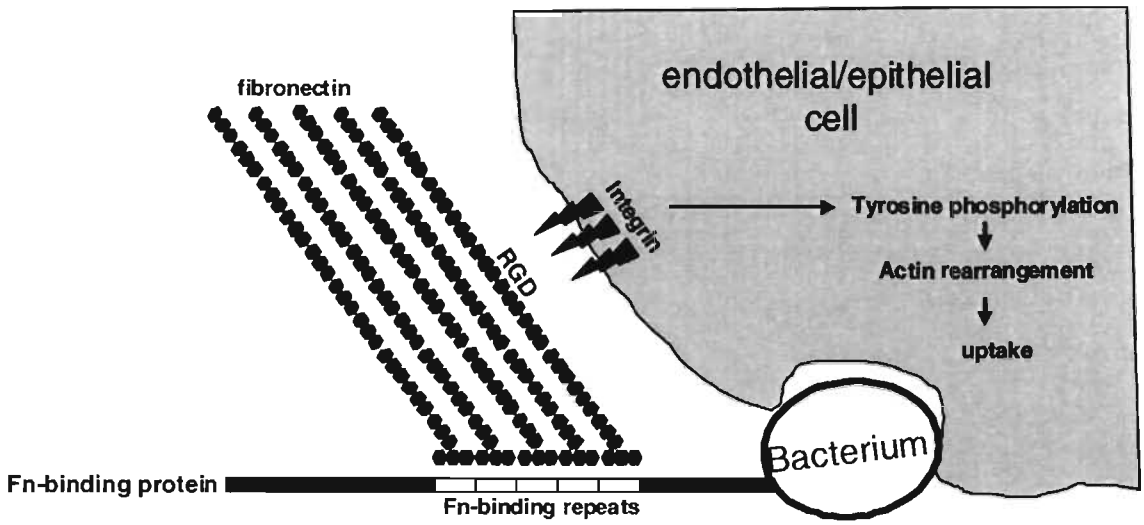

Fig 8. Schematic model indicating how binding of multiple Fn molecules to FnBPs attached to the bacterial cell surface might result in integrin clustering on the host cell surface and subsequent uptake of bacteria. Figure adapted from (373)

2.3.1. MSCRAMMS

The MSCRAMMs exhibit structural features typical of other cell wall-anchored proteins of Gram-positive bacteria (177) (Fig 9). A putative signal sequence involved in transport of the proteins through the cytoplasmic membrane by the Sec-dependent transport of the protein is located at the N terminus of these proteins. Following the signal sequence, MSCRAMM contain a domain, called A domain, that up to now has no known biological activity and the B domain that contain the fibronectin-binding repeats. The C-terminus is composed of: (i) a conserved LPXTG motif which is required for accurate sorting and anchoring of the proteins to the cell wall; (ii) a hydrophobic membrane-spanning region; and (iii) a tail of positively-charged residues which remain in the cytoplasm.

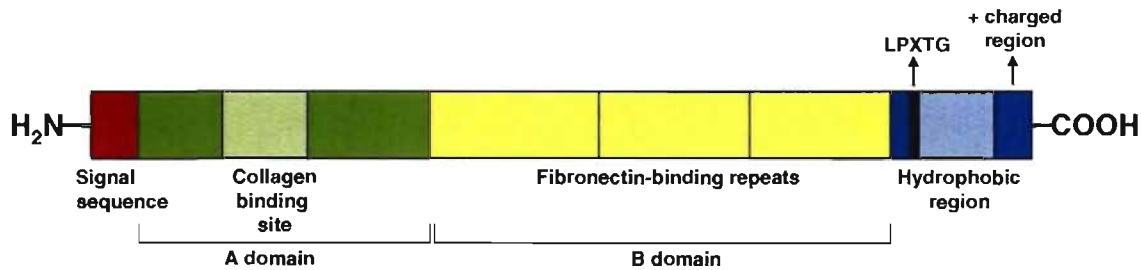

Fig 9: Diagram of the MSCRAMMs structure. Image adapted from (279)

Once the protein has been transported to the cell surface, the LPXTG motif is cleaved between the Thr and Gly residues. The carboxyl group of the Thr residue is then linked to a free amino group of a branched peptide within the peptidoglycan cell wall (326, 370). Finally, a proline-rich stretch of residues, thought to span the peptidoglycan layer of the cell wall, is commonly found on the N-terminal side of the LPXTG motif in these proteins. The enzyme responsible for catalyzing these reactions has been named 'sortase'. Sortase is a multifunctional protease-transpeptidase that has been shown to specifically cleave the LPXTG sequence in a protein and to catalyze the transfer of the processed protein to the free amino group of pentaglycine cross-bridges in peptidoglycans (277). Genes homologous to *srtA*, a sortase-encoding gene, are found in a variety of Gram-positive bacteria whose genome sequences have been entered in a database (235, 271), suggesting that the sortase-mediated mechanism is a universal strategy of Gram-positive bacteria for displaying proteins on the microbial surface. However, there is usually more than one *srtA* homolog in each genome, and the number of *srtA* homologs varies depending on the species (271). *S. suis* possesses five *srt* homologs: *srt*A encodes sortase, which anchors surface proteins with an LPXTG motif to the cell wall, while the functions of the other four homologs (the *srt*BCD cluster and *srt*E) remain unknown (267). The genetic organization of the srtA region was found to be conserved among *S. suis* strains (267). Although in some strains the *srt*As showed strong sequence divergence, their functions were verified to be overlapping by genetic complementation, indicating the functional conservation of *srt*As during the evolution of these strains (267). These results indicate the importance of a srtA-mediated cell wall sorting system for displaying proteins on the surface of *S. suis*.

2.3.2. Choline-binding proteins

Another mechanism of anchoring on the surface, distinct from LPXTG mediated attachment, is used by many surface proteins of different pathogens. Pneumococci have a series of surface-exposed proteins that attach to bacteria through this specific choline binding motif (36). The principal particularity of these proteins is that they do not possess the carboxy-terminal hydrophobic domain or charged tail, and also lack the LPXTG motif. These proteins, called choline-binding proteins, have modular structures consisting of a C-terminal choline binding module that is usually followed by a flexible linker peptide built from a proline-rich segment and finally by a functional N-terminal module (36). The choline binding module consists of several (usually around 10) repeat regions of usually approximately 20 amino acids. These modules have been identified to bind to terminal choline residues of teichoic or lipoteichoic acid structures present on the surface of *S. pneumoniae* and serves as an anchoring device to pneumococci for these proteins (36). The choline-binding protein motif has also been found among the surface proteins of other bacteria like *Clostridium acetobutylicum, Clostridium difficile, Streptococcus mutans*, and *Streptococcus downei* (403). However, up to now, no choline-binding protein has been described for *S. suis*.

2.3.3. Anchorless-proteins

Several Gram-positive cell-surface proteins with adhesive and invasive functions have been described that contain neither a signal peptide nor an LPXTG motif nor choline-binding repeats (65). The exact mechanism used by these proteins to be exported to the surface of bacteria is still unknown. However, a possible mechanism by which these anchorless proteins are displayed on the bacterial surface has been suggested by Bergmann *et al.* (37) who identified the anchorless surface-displayed α-enolase in *S. pneumoniae.* Although the described bacterial enolases have not the ability to bind fibronectin, it has been proposed that all the anchorless proteins, including the fibronectin-binding proteins, used a similar mechanism to be secreted to the surface of bacteria. Bergmann *et al.* suggested that this protein is secreted by autolysis and can reassociate by interacting with receptors on the surface of pneumococci. However, another study suggested that the secretion of the *Listeria monocytogenes* enolase is SecA2-dependent (213). Further research is required to identify the real mechanism used by these anchorless proteins to be secreted at the surface of the cell.

Fibronectin-binding anchorless proteins have biological functions as adhesins and invasins (65). Moreover, other biological activities have also been described for these proteins. For example, the *S. aureus* anchorless fibronectin-binding protein Eap (extracellular adherence protein) mediates diverse biological functions, including adherence and immunomodulation, thus contributing to *S. aureus* pathogenesis (144). Other example of these anchorless proteins is the fibronectin-binding protein PavA from *S. pneumoniae*, which was described by Holmes *et al.* (156). PavA is localized on the pneumococcal cell surface, despite the lack of conventional secretory or cell-surface-anchor signals. PavA binds to immobilized fibronectin and has been suggested to be involved in pneumococcal cell adhesion and to be essential for virulence (156). Another example is the fibronectin-binding protein FBP54 from *S. pyogenes*, which shows 67% homology to PavA, and neither contain the anchor features common to surface proteins of Gram-positive bacteria (73). As for the other described anchorless-fibronectin-binding protens FBP54 seems to play a role in the pathogenesis of the infection caused by *S. pyogenes*. Up to now, no fibronectin-binding anchorless protein of *S. suis* has been identified.

2.4. *S. suis* adhesion to ECM proteins

Although it has been demonstrated that ECM proteins are important host receptors for bacterial adhesins and that most of the bacterial ECM-binding proteins contribute to the pathogenesis of bacteria, little was known, before this work, about the capacity of *S. suis* to adhere to these host proteins. In fact, only three works have been published about the adhesion of *S. suis* to the proteins forming the ECM or about the *S. suis* adhesins involved in these processes.

The first one, realised by De Greef et al. in 1991, identified by "*in vivo selected genes*" the only fibronectin-fibrinogen-binding protein (FBPS) described for *S. suis* (78, 79). They showed that the *fbps* gene was present in all known serotypes of *S. suis* [except for serotypes 32 and 34 that up to now can be considered as *S. orisratti* (154)], as well as in all three phenotypes of serotype 2. However, the expression of FBPS in all serotypes and phenotypes was not studied (78). Therefore, it is possible that although all strains, except for serotypes 32 and 34, possess the *fbps* gene, not all strains express FBPS. The gene encoding FBPS was cloned and sequenced, and it has been showed that recombinant FBPS was able to adhere to human fibronectin and fibrinogen (78). In addition, using an isogenic mutant for this protein, it was demonstrated that FBPS is involved in the colonization of the organs specific for an *S. suis* infection in piglets, but not in the colonization of *S. suis* on the tonsils of piglets (78). The second work describes a 52 kDa IgG-binding protein (343). The characterisation of this protein, identified by Serhir *et al.* in 1993, demonstrated that it not only reacts with a large variety of IgGs and with human IgA, but also with other human plasma proteins including fibronectin (342). Finally, the last work about the adhesion of *S. suis* to fibrinogen has been recenty published (43). Bonifait *et al.* showed that supplementing the culture medium with fibrinogen induced biofilm formation by *S. suis* in a dose-dependent manner wich indicates that *S. suis* is able to bind fibrinogen to its surface and produce this biofilm (43). Moreover, this biofilm-grown *S. suis* were shown to be much more resistant to penicillin G than planktonic cells (43).

In addition to these adhesins, other proteins with high homology with other bacterial fibronectin-binding proteins have been described for *S. suis.* In 1993, the gene encoding the muramidase-released protein (MRP) was cloned and sequenced (348). Its analysis demonstrated that this protein contains a particular region within the amino acid sequence with high similarity with a fibronectin-binding protein of *S. aureus*. However, binding of MRP to human fibronectin could never be confirmed (348). In 2006, Baums *et al.*, also identified a gene of *S. suis*, named *ofs* (29), which encodes a protein with high homology to other streptococcal MSCRAMM fibronectin-binding proteins such as FnBA of *S. dysgalactiae* (237) and SOF of *S. pyogenes* (72). However, although OFS has a MSCRAMM typical structure, no detection of any fibronectin-binding activity was found for the recombinant OFS (29). The lack of detectable binding might be explained by significant differences between the repetitive sequences of OFS and the fibronectin-binding repeats of FnBA (237) and SOF (29) which are considered as the fibronectin-binding structure of these proteins.

3. Enolase

3.1. Glycolysis

3.1.1. Introduction

Glycolysis is a metabolic pathway by which a 6-carbon glucose (Glc) molecule is oxidized to two molecules of pyruvic acid (Pyr) (257). The word glycolysis is derived from Greek *γλυκύς* (sweet) and *λύσις* (rupture). It is the initial process of most carbohydrate catabolism, and it serves three principal functions: i) the generation of high-energy molecules (ATP and NADH) as cellular energy sources as part of anaerobic and aerobic respiration, ii) production of pyruvate for the citric acid cycle as part of aerobic respiration and iii) the production of a variety of six- and three-carbon intermediate compounds, which may be removed at various steps in the process for other cellular purposes (257).

As the foundation of both aerobic and anaerobic respiration, glycolysis is the archetype of universal metabolic processes known and occurring (with variations) in many types of cells in nearly all organisms. Glycolysis, through anaerobic respiration, is the main energy source in many prokaryotes, eukaryotic cells devoid of mitochondria (e.g. mature erythrocytes) and eukaryotic cells under low oxygen conditions (e.g. heavily exercising muscle or fermenting yeast) (257).

In eukaryotes and prokaryotes, glycolysis takes place within the cytosol of the cell. In plant cells some of the glycolytic reactions are also found in the Calvin-Benson cycle which functions inside the chloroplasts. The wide conservation includes the most phylogenetically deep rooted existant organisms and thus it is considered to be one of the most ancient metabolic pathways (309).

3.1.2. History

The most common and well-known type of glycolysis is the Embden-Meyerhof pathway. The first formal studies of the glycolytic process were initiated in 1860 when Louis Pasteur discovered that microorganisms were responsible for fermentation (329), and in 1897 when Eduard Buchner found that certain cell extracts could cause fermentation (189). The next major contribution was from Arthur Harden and William Young in 1905 who determined that a heat sensitive high molecular weight subcellular fraction (the enzymes) and a heat insensitive low molecular weight cytoplasm fraction (ADP, ATP and NAD^+ and other cofactors) were required together for fermentation (194). The details of the pathway itself were determined by 1940, with a major input from Otto Meyerhof (194) and some years later by Louis Leloir (193). The biggest difficulties in determining the intricacies of the pathway were due to the very short lifetime and low steady-state concentrations of the intermediates of the fast glycolytic reactions.

3.1.3. Glycolysis phases (257)

The breakdown of the six-carbon glucose into two molecules of the three-carbon pyruvate occurs in ten steps (Fig 10), the first five of which constitute the *preparatory phase*. In these reactions, glucose is first phosphorylated at the hydroxyl group on C-6 (step 1). The glucose 6-phosphate thus formed is converted to fructose 6-phosphate (step 2), which is again phosphorylated, this time at C-1, to yield fructose 1,6-bisphosphate (step 3). For both phosphorylations, ATP is the phosphoryl group donor. Fructose 1,6-bisphosphate is split to yield two three-carbon molecules, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (step 4); this is the "lysis" step that gives the pathway its name. The dihydroxyacetone phosphate is isomerized to a second molecule of glyceraldehyde 3-phosphate (step 5), ending the first phase of glycolysis. In this preparatory phase of glycolysis the energy of ATP is invested, raising the free-energy content of the intermediates, and the carbon chains of all the metabolized hexoses are converted into a common product, glyceraldehyde 3-phosphate.

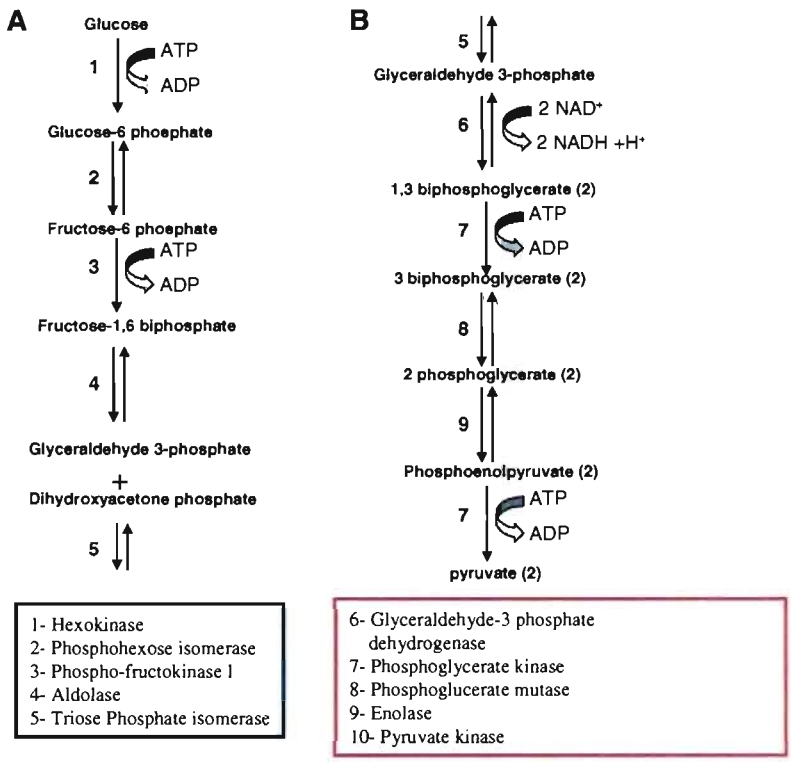

Fig 10. The two phases of glycolysis. Figure adapted from (257)

The energy gain comes in the *payoff phase* of glycolysis. Each molecule of glyceraldehyde 3-phosphate is oxidized and phosphorylated by inorganic phosphate (not by ATP) to form 1,3-bisphosphoglycerate (step 6). Energy is then released as the two molecules of 1,3-bisphosphoglycerate are converted to two molecules of pyruvate (steps 7 through 10). Much of this

energy is conserved by the coupled phosphorylation of four molecules of ADP to ATP. The net yield is two molecules of ATP per molecule of glucose used, because two molecules of ATP were invested in the preparatory phase. Energy is also conserved in the payoff phase in the formation of two molecules of NADH per molecule of glucose.

3.2. Enolase

3.2.1. Introduction

Enolase was discovered in 1934 by Lohman and Mayerhof while they were studying the conversion of 3-phosphoglycerate to pyruvate in muscle extracts (219). The enzyme α-enolase (2-phospho-d-glycerate hydrolase, EC 4.2.1.11) catalyses the conversion of 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP) by elimination of 1 molecule of water in the catabolic direction in the second half of the Emden Mayerhoff-Parnas glycolytic pathway (257) (Fig 10 B). In the other direction, ocurring during gluconeogenesis, the same enzyme catalyses the reverse reaction, the hydration of PEP to PGA (257). To be highly active, enolase requires certain divalent metal ions. The natural cofactor for this enzyme is magnesium. In presence of magnesium, enolase imparts its high activity, which classify this enzyme among the enzymes called metal-activated metalloenzymes (402). Enolase reaction also occupies a key position in the metabolic pathway of fermentation in general and the glycolytic pathway, in particular, and hence this enzyme is ubiquitously present in abundance in the biological world (402).

3.2.2. Distribution

All enolases are composed of two identical subunits, and have a molecular weight in the range of 82–100 kDa (272). In mammals and humans, there are three independent genetic loci, α, β and γ, that encode for three isozymes: i) α-enolase, that is found in a variety of tissues including liver, ii) β-enolase, that is almost exclusively found in muscle tissues and finally iii) γ-enolase, that is found in neuron and neuroendocrine tissue (282). Several earlier studies have determined from their chromatographic and immunological studies that neuron/brain-specific enolase can be found in heterodimeric forms such as $\alpha\alpha$, $\alpha\beta$, $\beta\beta$, $\alpha\gamma$ and γγ (108, 181, 312). As yet βγ heterodimeric isoenzyme has not been found, although experimentally it has been isolated from its individual homodimeric counterparts (306). Rider and Taylor (305, 306) and Merkulova et al. (241) described that the proportions of isoenzymes $\alpha\alpha$, $\beta\beta$ and $\alpha\beta$ in heart and skeletal muscle of rat varies during embryonic development. In both tissues, $\alpha\alpha$ isoenzyme predominates in the fetus stage however this isoenzyme is replaced by $\alpha\beta$ and $\beta\beta$ types in adult heart and by $\beta\beta$ type in adult muscle. These researchers also showed that the $\alpha\alpha$ isoform is found in liver, brain, spleen, adipose tissue and kidney.

3.2.3. Primary and secondary structure of α-enolase

It has been known since 1971 that α-enolase exist in a dimeric form (351). This structure was confirmed in the 80's and early 90's by X-ray crystallography. The results corroborate the dimeric form of this enzyme in which both identical subunits are facing each other in an antiparallel fashion. Each subunit of enolase is made up of two domains: the smaller N-terminal and the larger C-terminal domains (351).

Crystal structure of α-enolase from *S. pneumoniae* suggests that this enolase, and probably all the bacterial enolases, presumably form an octamer instead of a dimer under all physiological conditions, whether cytoplasmically located or presented on the surface of the bacterium (91).

3.2.4. Enolase activity

Warburg and Christian (388) and Utter and Werkman (374) observed for the first time the ion requirement of this enzyme. Since then, several groups have studied the importance and mechanisms of metal ion activation of the enolase enzyme (402). Six divalent metal ions – magnesium, manganese, zinc, cadmium, cobalt and nickel – were found to activate enolase (50). Among these, although magnesium is a naturally occurring metal activator, the binding of enolase with magnesium is much weaker than with zinc (50). Thus, according to the definition of Valle, enolase qualifies as a metal-ion-activated enzyme complex rather than a typical metalloenzyme in which the metal is firmly bound. The role of Mg^{2+} in the enolase activity is twofold since it possesses two types of binding sites which contribute to catalysis (99). The first site is called conformational. It induces conformational changes in the active site and enables binding of a substrate or its analogues. Once firmly bound, the second magnesium ion binds to the second site and serves as a required component of the catalytic apparatus. The relative activation strength profile of binding of metal ions in the enolase activity is $Mg^{2+} > Zn^{2+} > Mn^{2+} > Fe(II)^{2+} > Cd^{2+} > Co^{2+}$, Ni^{2+}, Sm^{3+}, Tb^{3+} (50, 402). Irrespective of which of these metal ions binds, the studies on yeast enolase have shown that metal ion binding in the catalytic site is always weaker when compared with that in the conformational site. Thus, although Ca^{2+}, Tb^{2+} and Sm^{3+} do not activate enolase, they bind to enolase much tighter than Mg^{2+} (49).

3.2.5. Mechanism of enolase activity (272)

Enolase catalyzes the elimination of OH^- from C3 of a discrete enolate intermediate which is created by removal of a proton from C2 of 2-PGA by a base in the active site in a stepwise manner (209).

When 2-PGA binds to α-enolase, it interacts with Gln167, Lys396 and both essential divalent cations (Mg^{2+}), and as a result the carboxyl group of PGA is rotated and neutralized. A large movement of the loop Ser36-His43 (36Ser-Gly-Ala-Ser-Thr-Gly-Val-His43) and a smaller movement of the loops Ser158–Gly162 and Asp255–Asn256 then result into the coordination of

backbone carbonyl and side-chain hydroxyl of Ser39 with catalytic Mg^{2+}. This movement appears to close the active site. The loops, Val153–Phe169 and Ser250–Gly277, then move together, allowing His159 to donate a proton to the phosphoryl of PGA. The interaction of protonated phosphoryl of PGA with Arg374 and catalytic Mg^{2+} then results in overneutralization of the negative charge on phosphoryl and the lowering of and hence bringing of carbon-2 pKa of PGA into the physiological range. The movement of Lys 345 closer to Arg374 lowers the pKa of its ε-ammonio group, which loses its proton and accepts one from the C2 of PGA. Finally, the proton shared by Glu168 and Glu211 forms a hydrogen bond to the PGA hydroxyl group. This proton is then transferred from the carboxylate to the hydroxyl group, making H_2O and PEP. In the reserve reaction (hydration reaction), the water held by Glu168, Glu211 and His373 is deprotonated by one of the carboxylates, and the resulting OH^- adds to C3. At the same time, a proton from the ε-ammonio group of Lys345 adds to C2, resulting in the elimination of the double bond between C2 and C3 (209).

3.2.6 Enolase superfamily

The Enolase superfamily is a paradigm of superfamilies whose members catalyse different reactions retaining a common mechanistic strategy. This superfamily was discovered by Babbitt *et al.* on the basis of the fundamental structure and the conserved amino acid residues of the catalytic site (23). Enolase superfamily comprises 16 enzymes which catalyze 11 different reactions. All the members of this family catalyze the initial metal-assisted abstraction of the α proton from a carbon adjacent to a carboxylate group, resulting in a stabilized enolate anion intermediate. The fate of this intermediate anion is then determined by the different active-site architecture, catalyzing a variety of chemical reactions that include racemization, cycloisomerization and β elimination of either water or ammonia using diverse carboxylate anions as substrate.

There are three subgroups in this superfamily, which is divided according to the basic residues involved in the formation of enolic intermediates (23): (i) an Enolase subgroup which utilizes a single lysine residue, (ii) a mandelate racemase subgroup (MR) which utilizes one lysine and/or a histidine residue(s) and (iii) a muconate lactonizing enzyme (MLE) subgroup which utilizes two lysine residues (138). Studies that recognize such a structure-function paradigm have provided a rational basis for understanding the chemistry of all of the superfamily members (22, 23).

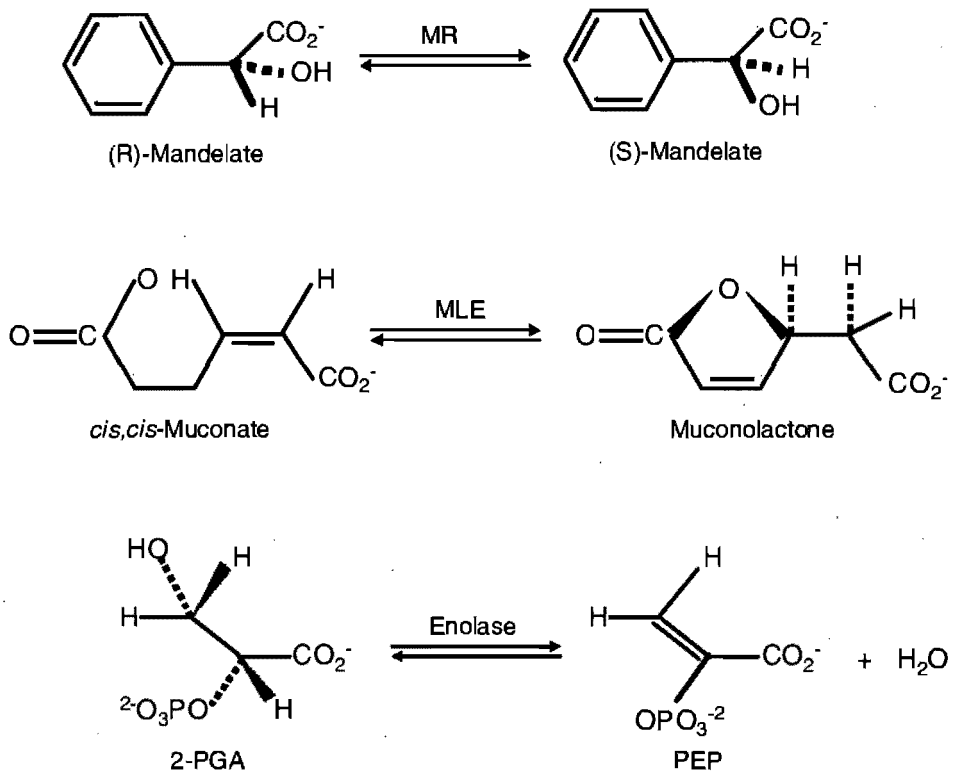

Fig. 11. Reactions of the enolase superfamily. Figure adapted from (23)

3.2.7. Location diversity

3.2.7.1. Enolase as a cytosolic enzyme

As explained above, enolase was discovered for its participation in one of the steps of the glucolysis and gluconeogenesis. As both metabolic pathways take place within the cytosol of the cell (272), it was commonly believed that the localisation of this enzyme was only the cytoplasm of cells. However, through the years, it has been demonstrated that the localisation of this metabolic enzyme is not restricted to cytoplasm but it can also been found in the other cell compartments.

3.2.7.2. Enolase as a surface protein

Enolase was first described on the surface of a great variety of eukaryotic cells as a strong plasminogen-binding receptor (245, 256, 303). Later, the presence of enolase on the surface of prokaryotes as a plasminogen receptor was also reported in many pathogens and commensal bacteria (273). The expression of a glycolytic enzyme on the surface of cells is not unprecedented. Other enzymes such as GAPDH and phosphoglycerate kinase have also been reported on the surface of different cell types including prokaryotes (274). However, a challenging question remains about how these glycolytic enzymes, including enolase, are expressed on the surface of eukaryotic cells such as haematopoetic cells, epithelial cells, neuronal cells and also on Gram-positive cocci, *Candida* and parasites (272). The mechanism used for its translocation to the surface of cells is still unknown; however, it is important to remark that in almost all cases, the surface

localisation of these proteins belongs to organisms which possess only plasma membrane or a membrane and a cell wall, but not organisms which possess two membranes, such as in Gram-negative bacteria.

The amino acid sequence of bacterial enolases shows that this enzyme lacks the N-terminal signal sequence and also the anchor carboxyl terminus LPXTG motif. These domains have been shown to be crucial for translocation through the cell wall and for anchoring surface proteins to the Gram-positive bacterial cell wall (104). Furthermore, repeats that might anchor the protein non-covalently to the surface, as shown for choline-binding proteins of *S. pneumoniae* (405) are neither present on bacterial enolases. The precise mechanisms of how enolases or other anchorless protein (273, 274) are transported through the cell wall and how these proteins are anchored to the bacterial cell surface need further investigation. One study suggested that the secretion of the *L. monocytogenes* enolase is SecA2-dependent (213). Another study speculated that surface GADPH and enolase are rather 'scavenged' from other cells that have undergone programmed cell death through allolysis (137). However, it remains unknown why these enzymes and, in particular, enolase appear on the surface of cells.

3.2.7.3. Enolase as a nuclear protein

In addition to the cytoplasmic and surface localisation of this enzyme, it has been recently demonstrated that enolase is also found in the nucleus in several cell types. Western blotting shows that this protein is present in both nucleus and cytoplasm of endothelial cells (1) and HeLa cells (357). Immuno-histochemistry also shows a nuclear location for α–enolase in astrocytes (201), type II neurons of the spiral ganglia (81), bronchial epithelial cells, type I and type II alveolar cells and endothelial cells (58).

It has also been demonstrated that during muscle regeneration α–enolase changes from a cytoplasmic to a perinuclear location suggesting a role of nuclear enolase during this regeneration (240). This hypothesis was also proposed when a recent study showed that in the parasite *Plasmodium yoelii*, a small fraction of enolase undergoes several posttranslational modifications, permitting this protein to switch from cytoplasm to nuclear localization (270). The variable amounts of enolase detected in nucleus at different life cycle stages of the parasite, also suggests that enolase could have some type of nuclear function depending on the stage of development of the parasite.

3.2.8. Multifunctional nature of enolase

Since enolase is abundantly expressed in most cells, it has been proved useful as a model for studying basic mechanisms of enzyme action as well as structural analysis. However, unlike the gene that expresses GAPDH, the gene that expresses enolase is not a housekeeping gene since its expression varies according to the pathophysiological, metabolic or developmental conditions of

cells (272). Several studies have reported different unrelated functions for enolase. The accumulating evidence makes clear that enolase is a multifunctional protein.

3.2.8.1. Enolase as a plasminogen-binding protein

In both eukaryotic and prokaryotic cells, cell-surface α-enolase serves as a strong receptor and activator of plasminogen, a key component of the intravascular and pericellular fibrinolytic systems (21, 272). In *S. pyogenes* infections, the binding of α-enolase to the cell-surface is associated with the loss of its glycolytic activity and the acquisition of a high affinity for plasminogen (111, 272). The surface-exposed epitope involved in this function is located in the central loop of human α-enolase (amino-acids 257–272) (21). The crystal structure study of α-enolase from *S. pneumoniae* reveals in fact the presence of two plasminogen binding sites. The first one matches the C-terminal lysyl residues but may not be as important as the second one since some α-enolases, including *C. albicans* α-enolase, entirely lack the first plasminogen binding site. In *S. pneumoniae*, the α-enolase binding to plasminogen induces plasminogen transformation into plasmin and is thought to be a virulence factor by preventing the generation of fibrin clots and thus enables tissue invasion, as described for *S. pyogenes* and *S. pneumoniae* (111).

3.2.8.2. Enolase as a laminin-binding protein

In addition of its plasminogen-binding activity, it has been demonstrated that enolase from *S. aureus* is also able to adhere to laminin, the major protein constituting the basement membrane of cells (54). This laminin-binding activity might function as a guidance mechanism, allowing *S. aureus* adherence to the basement membrane and initiating tissue colonization. Recently, it has been demonstrated that the enolase of the commensal bacteria *Lactobacillus crispatus* also adheres to laminin indicating that this is not an isolated feature of *S. aureus* and that this function could be biologically important for these bacteria (15).

3.2.8.3. Enolase as an eye τ-crystallin protein

The principal components of eye lens, contributing about 20–60% of total wet weight are the crystallins (286). The vertebral lenses are composed of α–, β–, γ–, δ–, ε– and *t*-crystallins (401). τ-crystallin is present in as much as 23 % of the total protein of the lens. Purified τ-crystallin sequence shows strong similarity to the sequences of human and yeast enolases indicating a commun origin for both proteins. However, in contrast with the glycolitic enolase, this protein shows significantly low enzymatic activity (399). Three different reasons seem to be the cause for this discrepancy in activity: i) it seems that during development and differentiation, the fiber cells of the lens lose all their organelles, and hence they must rely on cytoplasmic glycolysis as a source of energy (400), ii) as described above, enzymatically active enolase is found in dimeric form (402), however, τ–crystallin is redominantly found in monomeric losing its activity and finally iii) it is

also believed that the loss of enzyme activity may be due to aging in the lens or the presence of some inhibitor substance(s) in the lens (399).

3.2.8.4. Enolase as a Myc-binding protein (MBP-1)

C-myc is a DNA-binding phosphoprotein protooncogene that plays a crucial role in the regulation of cell growth and differentiation (231). The human c-myc protooncogene contains two TATA boxes separated by 165 bp located at the 5'end of the first axon (287). MBP-1 is a 37-kDA protein that is able to bind in a region +123 to +153 relative to the c-myc P2 promoter (301). MBP-1 is a negative regulator of c-myc expression because it binds in the minor groove of the c-myc P2 protein simultaneously with TATA-binding protein impeding the transcription of the gene (101, 231). It has been proved that binding of MBP-1 to c-myc leads to tumor suppression.

The sequence analysis of MBP-1 has shown that there is extensive homology between this gene and the gene coding for α-enolase (357). Insterestingly, the start codon MBP-1 ATG corresponds to the 400 bp downstream of the α-enolase ATG, and hence MBP-1 is considered to be an alternative translation initiation product of the α-enolase RNA (357). Since MBP-1 lacks the first 96 residues of α-enolase, it does not show enolase activity, suggesting that the N-terminal portion is essential for glycolytic activity, and is not essential for c-myc binding. However, studies using a mutant enolase protein lacking amino acid residues 96–236, showed that this mutant no longer binds to DNA, suggesting that the N-terminal portion of MBP-1 is involved in c-myc binding for the downregulation of its expression and tumor suppression (101).

3.2.8.5. Enolase as an endothelial hypoxic stress protein

In adapting to extreme environments such as high temperature or glucose deprivation, cells often secrete or express specific proteins known as heat-shock proteins (HSPs) (406). Vascular endothelial cells in a similarly stressful situation such as chronic hypoxia respond by upregulating the expression of a unique set of five cell-associated stress proteins (HAPs) (*Mr* 34, 36, 39, 47 and 57) in a time- and oxygen concentration-dependent manner (133). One of these proteins, the 47-kDa protein, has been identified as α-enolase. These results proposed that, in a hypoxic situation, upregulation of enolase may provide protection to cells by increasing anaerobic metabolism (1).

3.2.8.6. Enolase as a heat shock protein (HSP)

In *Saccharomyces cervesiae* several HSPs are induced at elevated temperatures. One of these, HSP48, has been identified as α-enolase (164). HSP48 is the product of the *eno* gene and is thought to be involved in both thermal tolerance and growth control in this organism. The expression of the *eno* gene is negatively regulated by the *HSR1* (*heat shock resistant*) gene since the *HSR1* mutant renders the organism extremely resistant (1000-fold more resistant then the parental strain) to heat shock (164).

3.2.8.7. Enolase as microtubule organizer

Recently, several studies have indicated that glycolytic enzymes are anchored to specific substructures (188). In fact, adhesion of enolase to microtubules has been reported by several groups. Such mechanism appears to occur, for example, in sperm flagellae, where the association of glycolytic α-enolase to microtubules is likely necessary for human sperm motility (119). In a similar way, the high ATP turnover rates, needed in *Chlamydomonas* for flagellae motility, are supported by the presence of enolase in the flagellar microtubular compartment (248). The conservation during evolution of this enolase/microtubule interaction most probably reflects a fundamental, indispensable function of these molecular associations.

Moreover, it has also been proposed that enolase could play a role in the dynamic cytoskeletal re-organization accompanying myogenesis both *via* its direct binding to tubulin/microtubule system and *via* glycolytic energy production (183). The co-localisation of enolase with the microtubular system was demonstrated in undifferentiated myoblasts. It seems that under this condition, the tubulin formed by depolymerisation of microtubules, could be complexed with the enolase. One can, therefore, speculate that the multiple associations of enolase with tubulin/microtubules could influence effectively the dynamics of the microtubular network and could contribute to regulate the reorganisation of cytoskeleton known to occur during myogenesis (183).

3.2.9. Enolase and disease

Antibodies against α-enolase have been detected in a large variety of infectious and autoimmune diseases. These antibodies might arise as a consequence of a microbial infection or uncontrolled growth or proliferation of cells in specific organs under pathophysiological conditions.

3.2.9.1. Anti-α-enolase antibodies in infectious diseases

A major contributing factor in the development of antibodies against bacterial antigens is the fact that several antigens may share common epitopes with the antigens present on the surface of other bacteria. This is especially true for enolase which has epitopes that are able to ross-react with different species of streptococci and the human enzyme (313). Despite the differences found in species, α-enolase is highly conserved, with a 40 to 90% identity between enolases from two different species (272). For example, Group A *Streptococcus* and *Saccharomyces cerevisiae* enolases share 43% and 62% homology with human α-enolase, respectively.

Antibodies against *S. pneumoniae* α-enolase develop early in life. Adrian *et al.*, in a cohort study of children presenting with otitis media during their first two years of life, detected antibodies against *S. pneumoniae* α-enolase in 99% of the sera (6). The authors suggest that the high prevalence of anti-α-enolase antibodies in children may be due to cross-reactive epitopes of α-enolase from colonizing bacterial species because of the high degree of homology of α-enolase

among these species (6). Anti- α-enolase antibodies, along with other anti-neuronal glycolytic enzymes, have also been detected more frequently in patients with post-streptococcal neuropsychiatric sequelae as compared to controls (75). Anti-α-enolase antibodies could also play a role in limiting tissue invasion in streptococcal infections as shown by partial inhibition of plasminogen binding to pharyngeal cells in the presence of polyclonal anti-*S. pyogenes*-α-enolase antibodies (272). Finally, anti-streptococcal enolase monoclonal antibodies inhibit phagocytosis of the bacteria by human neutrophils (111). Antibodies against *C. albicans* α-enolase are also present in the sera of immunocompetent subjects in case of colonization with *C. albicans*. For immunodeficient patients, the rates of antibodies against *C. albicans* α-enolase are significantly higher in the sera of patients with invasive candidiasis than in the sera of patients colonized with *C. albicans* and anti-α-enolase antibodies can thus be used as a diagnostic test for systemic candidiasis (377).

3.2.9.2. Anti-α-enolase antibodies in systemic autoimmune disorders

Anti-α-enolase antibodies have been found in a large variety of autoimmune and inflammatory diseases. Anti-α-enolase antibodies have been initially reported in sera from patients that reacted with centrosomes in systemic rheumatic diseases (300). Then, anti-α-enolase antibodies have been shown to be a minor target antigen of anti-neutrophil cytoplasm antibodies (ANCA) in systemic vasculitides (250), ulcerative colitis and Crohn's disease (310) and primary sclerosing cholangitis (266). Since then, anti-α-enolase antibodies have also been found in a large variety of autoimmune and inflammatory disorders such as systemic lupus erythematosus (SLE) (288), mixed cryoglobulnemia (MC) (288), systemic sclerosis (SSc) (288), rheumatoid arthritis (RA) (323), Behçet's disease (211), multiple sclerosis (212), Hashimoto's encephalopathy (HE) (114) and paraneoplastic retinopathy such as cancer-associated retinopathy (CAR) (5). In healthy controls, antibodies against α-enolase have been found in 0 to 6% (266)(288) (5). The production of anti-α-enolase antibodies is not isotype restricted (288).

Taking into account the wide spectrum of diseases associated with anti-α-enolase antibodies, these antibodies cannot help in the diagnosis of a specific autoimmune disease. However, anti-α-enolase antibodies could be used as prognostic markers. In SLE and MC, the presence of anti-α-enolase antibodies was associated with renal involvement (250, 288, 313) although this remains controversial. In SSc, the number of patients tested was small and no correlation was clearly made between the detection of anti-α-enolase antibodies and clinical manifestations, except for severe organ involvement (288). In RA, the presence of anti-α-enolase antibodies was associated with radiological progression (323). In HE, high titers of anti-α-enolase antibodies were associated with an excellent corticosteroid sensitivity (114). In primary biliary

cirrhosis, no correlation was found between the presence of anti-α-enolase antibodies and clinical presentation, but the mortality rate associated with hepatic failure was significantly higher in patients with anti-α-enolase antibodies (8).

The titer of anti-α-enolase antibodies is high in systemic diseases, and relatively low in liver diseases, suggesting that the level of Ab affinity could play an important role, as well as differences in terms of Ab specificity. Indeed, the identification of reactivities with the different enolase isoforms (α, β, γ) in various autoimmune diseases suggest the recognition of different epitopes of enolase (288). Moreover, reactivities between purified and recombinant α-enolase differ, suggesting the predominant recognition of post-translationally or post-traductionally modified forms of α-enolase, such as citrullination (186). For example, the majority of sera tested in SLE, MC or HE expressed Ab reactivity toward α-enolase, whereas more than half of sera from patients with CAR (5) and lymphocytic hypophysitis (262) reacted either with α- or γ–enolase.

Epitope-mapping was performed in CAR and in endometriosis. Four major epitopes of α-enolase (amino-acids 31–38, 176–183, 421–428 and 56–63) were found to be recognized by all anti-α-enolase antibodies of CAR patients, the epitope 56–63 being specifically associated with pathogenic sera (4). Interestingly, sera from healthy subjects recognized the epitopes 30–37, 176–183 and 421–428 of α-enolase but not the epitope 56–63 (4). In endometriosis, anti-α-enolase antibodies bound preferentially to two epitopes (53–87 and 207–238) (387), and shared the reactivity against the epitope 56–63 with CAR patients.

3.2.9.3. Pathogenic role of anti-α-enolase antibodies

Anti-α-enolase antibodies might be produced after a contact with bacteria or yeast and cross react with human-α-enolase (288) Indeed, Fontan *et al.* suggested that antibodies raised against streptococcal α-enolase during *S. pyogenes* infections might recognize common epitopes of human α-enolase expressed at the membrane of eukaryotic cells and could be involved in post streptococcal sequelae (111). Interestingly, higher titers of anti-streptococcal α-enolase antibodies are found in serum samples from patients with acute rheumatic fever as compared with healthy controls or patients with minor pharyngitis. The potential role of an immune response directed against yeast was supported by the binding of anti-α-enolase antibodies from patients with autoimmune hepatitis and primary sclerosing cholangitis to *Saccharomyces cerevisiae* enolase. Also, in patients with a *C. albicans* infection, yeast enolase is released (358) and may generate an immune response. Finally, particular immune background may play a role since it was shown that human α-enolase-derived peptides bound to HLA-DR8, suggesting the occurrence of autoimmune diseases in patients with HLA-DR8 allele (66).

The role of anti-α-enolase antibodies has been extensively studied in patients with SLE related-nephritis and CAR. In patients with SLE related-nephritis, α-enolase was found to be overexpressed in glomeruli and inflammatory lesions such as crescents, whereas in normal kidney, α-enolase was almost undetectable in glomeruli and expressed only in tubuli (244). Since the upregulation of α-enolase can increase the tolerance to hypoxia (272), anti-α-enolase antibodies might be involved in the pathogenesis of SLE related-nephritis and act as a nephrogenic autoantigen. BALB/c mice were injected intraperitoneally with hybridoma-producing anti-α-enolase antibodies, and some of the glomeruli showed focal infiltrates or diffuse proliferative lesions. Thus, since α-enolase is expressed on the membrane of various cells, anti-α-enolase antibodies could play a role in renal and endothelial injury through the generation of immune complexes and activation of the complement classical pathway (288)

Anti-α-enolase antibodies can inhibit the binding of plasminogen to α-enolase (251), and thus plasminogen activation to plasmin. As reported in SLE, in MC and SSc, anti-α-enolase antibodies react with endothelial cells expressing large amounts of α-enolase, and induce injury to those cells. Moreover, anti-α-enolase antibodies isolated from the serum of patients with SLE, MC or SSc bound to the membrane-associated form of the enzyme interfering with the plasminogen receptor function (251). In addition, although hematopoietic cells display several molecules that bind plasminogen, α-enolase is responsible for the majority of the promotion of plasminogen activation on their surface, and a monoclonal anti-α-enolase Ab inhibits the cell-surface mediated plasminogen activation (220).

In CAR patients, anti-α-enolase antibodies are capable of penetrating retinal tissue to target ganglion cell layer and inner nuclear layers and, consequently, induce death of retinal cells through an apoptotic process (304). Cytotoxicity of CAR antibodies occurs independently of complement activation and is dependent on the amount of antibodies added and the duration of exposure of retinal cells to these antibodies. Moreover, when anti-α-enolase antibodies were injected intravitreously into rat eyes, these antibodies also targeted retinal cells in the inner nuclear layer and ganglion cell layer, and consequently caused apoptotic cell death (304). Interestingly, it was reported that anti-α-enolase antibodies from patients with CAR, patients with endometriosis or healthy individuals did not bind to the epitope 257–272, involved in the plasminogen-binding of α-enolase, suggesting that these conditions are not associated with perturbations of the intravascular and pericellular fibrinolytic system (304).

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III. MATERIAL, METHODS AND RESULTS

ARTICLE I

Streptococcus suis serotype 2 Binding to Extracellular Matrix Proteins

Miriam Esgleas, Sonia Lacouture and Marcelo Gottschalk*

Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP), Faculté de médecine vétérinaire, Université de Montréal, Québec, Canada and Canadian Research Network on Bacterial Pathogens of Swine

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I am the main author of this article.

ABSTRACT

Streptococcus suis serotype 2 is a major swine and human pathogen that causes septicemia and meningitis. The ability of *S. suis* serotype 2 to bind to different extracellular matrix (ECM) proteins was evaluated by ELISA. All 23 strains tested bound to plasma and cellular fibronectin and collagen types I, III, and V, some to fibrin, vitronectin, and laminin, and none to the other ECM proteins tested. An unencapsulated isogenic mutant bound to ECM proteins better than its parental encapsulated strain, suggesting that the polysaccharide capsule interfered with binding. Cross-inhibition was observed between soluble plasma fibronectin and collagens in the ECM adherence assay, indicating that binding domains for both proteins exist on the same or nearby bacterial surface molecules. On the other hand, preincubation with plasma fibronectin increased binding to collagen IV, suggesting that *S. suis* might use fibronectin as a bridging molecule. The results of heat treatment and proteolytic digestion suggest that adhesins for these ECM proteins are proteinaceous in nature.

INTRODUCTION

Streptococcus suis is a major swine pathogen that causes septicemia, meningitis, endocarditis, and arthritis (17). Of the 35 known serotypes, serotype 2 is most frequently isolated and associated with diseases (17). This pathogen is also a zoonotic agent that causes similar diseases in humans (2, 20, 35).

Little is known about *S. suis* virulence factors. The capsule polysaccharide (CPS) is the only proven virulence factor since unencapsulated isogenic mutants are completely avirulent and are rapidly cleared from circulation in pig and mouse infection models (9, 33). Other putative virulence factors include a hemolysin (suilysin), a 136-kDa muramidase-released protein (MRP), a 110-kDa extracellular factor (EF) protein, a hyaluronidase, a superoxide dismutase, various proteases, and bacteriocins (29).

The extracellular matrix (ECM) is a stable macromolecular structure underlying epithelial and endothelial cells and surrounding connective tissue cells (41) that becomes exposed when tissue integrity is disturbed by lesions or traumas. The ECM is composed of glycoproteins and glycosaminoglycans (GAG). Its composition differs in various organs, but the main components are fibronectin, collagen, elastin, laminin and GAG, like heparin, and heparan sulfate (21). Many of these proteins can serve as potential cell receptors for bacteria and participate in the infectious process (13, 19, 24, 28, 41). In addition, bacterial binding to plasma proteins such as fibronectin and fibrinogen can prevent recognition of bacteria by the host immune system by masking immunogenic epitopes (12).

Binding of virulent bacteria to host cells is the first step in the colonization of mucosal surfaces. Binding may also be a first step in the invasion of host cells, a process that may lead to bacteremia and sepsis (36). The pathogenesis of infections caused by *S. suis* is not fully understood and many steps are probably involved (15). *S. suis* is able to bind to and, in some cases, invade endothelial and epithelial cells of human and porcine origin (4, 15, 22, 26, 39). However, the mechanisms involved in these interactions are unknown.

While various streptococci specifically bind to host ECM and while these interactions play a role in disease pathogenesis, little is known about the ability of *S. suis* to bind to ECM proteins. Recently, a fibronectin-fibrinogen-binding (FBPS) protein has been proposed as a contributing factor in the colonization of organs due to its binding to ECM proteins of host cells (11). However, interactions between *S. suis* and various ECM proteins have never been studied. The purpose of this study was to evaluate the ability of *S. suis* serotype 2 field strains to specifically bind to various ECM proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S. suis serotype 2 strain S735 (ATCC 43765) was used as the reference strain. An unencapsulated mutant strain (B218) obtained in our laboratory by allelic exchange [22] and corresponding to a transposon-derived mutant described previously (14) and its virulent parental strain (31533) were also included. Strain B218 was shown to possess the same characteristics that mutant 2A, with a complete absence of capsular material at the bacterial surface. In selected experiments, 23 field strains of *S. suis* serotype 2 were used. These strains originated from different species (human or swine), different pathologies and different geographical origins. A complete list is presented in Table 1. Strains were grown as previously described (1). Late exponential-phase bacteria were washed three times in phosphate-buffered saline (PBS) (pH 7.3) and killed by suspending them in 0.2% (v/v) formaldehyde overnight at 4°C.

Chemicals

Commercially available human ECM proteins were used. Cellular fibronectin, collagen types III and V, laminin, plasma vitronectin, plasma fibrin, and fibrinogen were from Sigma Chemical Co. (St. Louis, MO, USA); plasma fibronectin was from Roche Diagnostics Corporation (Indianapolis, IN, USA); collagen types I and IV were from BD Biosciences (Bedford, MA, USA); and elastin was from EMD Biosciences Inc. (La Jolla, CA, USA). Porcine fibrinogen was from Sigma, pronase and proteinase K were from Roche, and trypsin was from Gibco (Burlington, ON, Canada).

Microtiter plate binding assay

Maxisorp® flat-bottom microtiter 96-well plates (Nunc, VWR, Mississauga, ON, Canada) were coated with 100 µl (0 to 50 µg ml^{-1}, depending on the experiment) of ECM protein (plasma fibronectin; cellular fibronectin; collagen types I, III, IV, and V; laminin, elastin, vitronectin, fibrin, human fibrinogen; and porcine fibrinogen) in 0.1 M carbonate coating buffer (pH 9.6) and were incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST, pH 7.3), and 200 µl of 3% (w/v) non-fat dry milk in PBST was added to each well to prevent non-specific bacterial binding. After 1 h at 37°C, the wells were washed three times with PBST. Formaldehyde-killed bacterial suspensions (100 µl) of individual strains were added and the plates were incubated for 2 h at 37°C. Different bacterial concentrations, incubation times, and temperatures were also tested. All unbound bacteria were subsequently removed by washing the wells three times with PBST. A 100 µl volume *S. suis* serotype 2-specific rabbit antiserum

(diluted 1/3,000 in PBST) prepared as previously described (16) was then added to each well. The plates were incubated for 1 h at 37°C. The wells were washed three times with PBST and 100 µl of horseradish peroxidase-labelled anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) (diluted 1/8,000 in PBST) was added. The plates were incubated for 1 h at 37°C with the secondary antibody. After washing three times with PBST, 3,3',5,5'-tetramethylbenzidine (Zymed, San Francisco, CA, USA) was used as the enzyme substrate according to the manufacturer's instructions. The reactions were stopped by adding 25 µl of H_2SO_4 (1N) and were read at 450 nm using a microplate reader (Uvmax; 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.

Proteolytic, formaldehyde, and heat treatments

For the proteolytic treatment, 1 ml of live bacterial suspension (10^8 bacteria ml^{-1}) was centrifuged and resuspended in the same volume of PBS containing one of following enzymes: trypsin, pronase, or proteinase K. All proteolytic enzymes were used at a concentration of 1 mg ml^{-1}. The suspensions were incubated for 1 h at 37°C for trypsin and proteinase K and 40 minutes for pronase. Controls with either an untreated suspension of live bacteria or 0.2% formaldehyde-treated bacteria were also incubated for 1 h at 37°C. The suspensions were then washed three times in PBS and resuspended in 1 ml of PBS for use in microtiter plate binding assay. The heat sensitivity of the components involved in the adhesion of bacteria to the ECM proteins was evaluated by heating bacterial suspensions for 30 min at 60°C or 100°C.

Inhibition assays

For the inhibition assays, bacterial suspensions were preincubated for 120 min with 150 µg ml^{-1} of various ECM proteins (plasma fibronectin, cellular fibronectin, collagen type I, collagen type III, or collagen type V) before being incubated with immobilized fibronectin, collagen, or fibrin. After the preincubation period, the suspensions were centrifuged to remove unbound protein, the bacteria were added to the wells, and the ELISA was performed as described above.

Statistical analysis

ELISA tests were performed at least three times for each binding assay. Differences between strains (31533 vs. B218), different treatments (proteolytic, formaldehyde, and heat treatments), and fibronectin-mediated binding (pre-incubation with fibronectin vs. pre-incubation with PBS) were analyzed for significance using general linear models followed by the Tukey-

Kramer post-hoc test for differences between the various incubation temperatures (4°C, 20°C, and 37°C).

Strain	Source	Geographical Origin
S735	Pig: meningitis	Europe
98-B575	Pig: endocarditis	Canada
99-1539B	Pig: endocarditis	Canada
98-C462B	Pig: endocarditis	Canada
98-8993	Pig: endocarditis	Canada
98-B099	Pig: meningitis	Canada
98-B719	Pig: meningitis	Canada
SS166	Pig: meningitis	Europe
D-282	Pig: meningitis	Europe
89-1591	Pig: meningitis	Canada
90-1330	Healthy pig	Canada
94-623	Healthy pig	Europe
24	Pig: septicemia	Europe
T15	Healthy pig	Europe
91-1804	Human: endocarditis	Canada
98-3634	Human: endocarditis	Canada
Biotype2/hemo	Human: endocarditis	Europe
Reims	Human: spondylodiscitis	Europe
94-3037	Human: meningitis	Europe
FRU95	Human: meningitis	Europe
LEF95	Human: meningitis	Europe
31533	Pig: meningitis	Europe
B218	Unencapsulated mutant derived from strain 31533	

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

RESULTS

Binding of *S. suis* serotype 2 reference strain S735 to ECM proteins

As shown in Figure 1, strain S735 bound to both fibronectins (plasma and cellular) and to collagen types I, III, and V. This strain did not bind to the other proteins tested (data not shown). No binding was observed in the casein-coated control wells, indicating that binding to the five ECM proteins was specific.

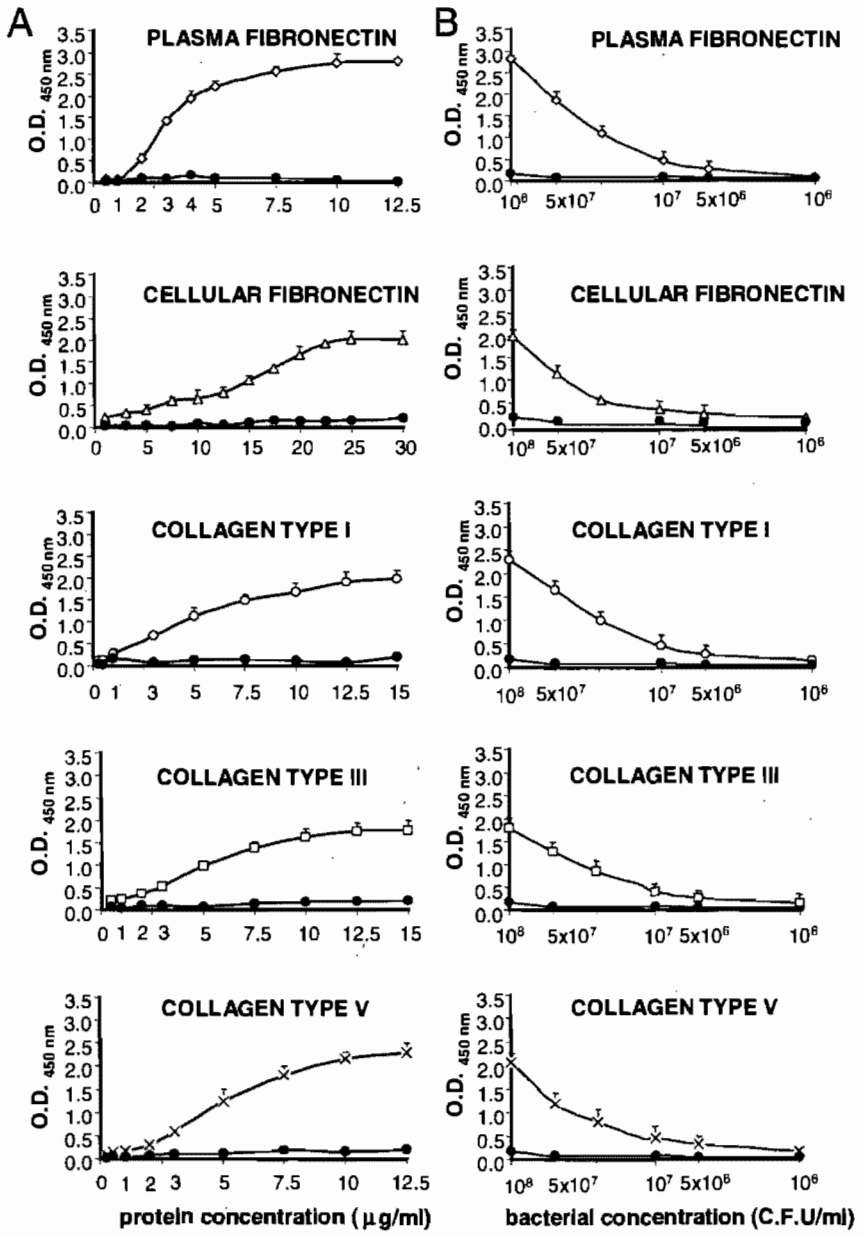

Fig. 1. (A) Effect of ECM protein concentrations on *S. suis* serotype 2 strain S735 binding. Plates were coated with different ECM protein concentrations (open symbols), bacteria were added at 10^8 bacteria ml^{-1}, and binding was evaluated after 2 h. (B) Plates were coated with fixed protein concentrations (10 µg/ml for plasma fibronectin, 25 µg/ml for cellular fibronectin, and 12.5 µg/ml for collagen types I, III, and V) (open symbols) and different bacterial concentrations (from 10^6 to 10^8 C.F.U ml^{-1}) were added. The plates were then incubated for 2 h. Casein (10 µg/ml; closed symbols) was used as a negative control. Data are expressed as means ± standard deviations of at least three experiments performed in triplicate.

Binding of *S. suis* serotype 2 reference strain S735 as a function of protein and bacterial concentration, time, and temperature of incubation

Adherence positively correlated with bacterial and protein concentrations. The binding of strain S735 increased with increasing protein concentration and reached a plateau at 10 μg ml^{-1} for plasma fibronectin, 25 μg ml^{-1} for cellular fibronectin, and 12.5 μg ml^{-1} for collagen types I, III, and V (Fig. 1A). However, even with these proteins, no binding was detected with bacterial concentrations under 10^7 bacteria ml^{-1} (Fig. 1B).

Strain S735-binding to both fibronectins and collagen types I and V reached half-maximum between 30 and 60 min and maximum binding at 120 min, while binding to collagen type III reach half-maximum between 60 and 90 min and maximum at 120 min. (Fig. 2). Similar results were obtained with the non-encapsulated strain B218 (result not shown).

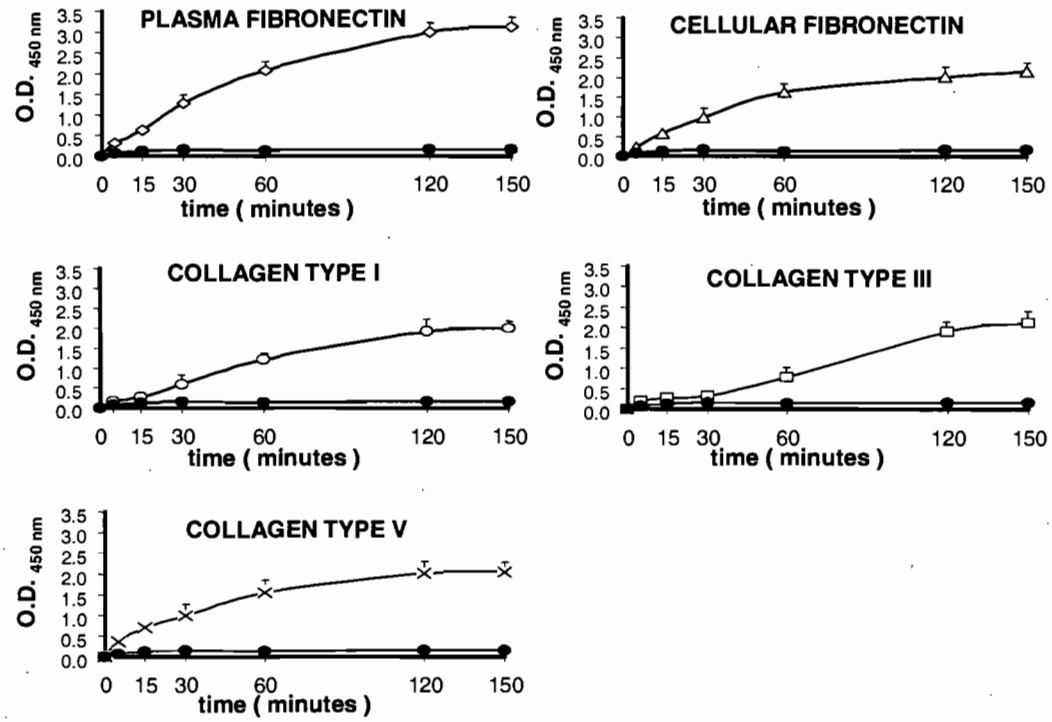

Fig. 2. Time course of *S. suis* serotype 2 strain S735-binding to different ECM proteins (open symbols). Wells were coated either with 10 μg/ml of plasma fibronectin, 25 μg/ml of cellular fibronectin, or 12.5 μg/ml of collagen types I, III, or V and incubated with bacteria (10^8 C.F.U ml^{-1}). Data are expressed as means ± standard deviations of at least three experiments performed in triplicate. Casein (10 μg/ml; closed symbols) was used as a negative control.

Strain S735-binding to both fibronectins and collagen types I, III, and V was also measured at different temperatures (4°C, 20°C, and 37°C). As shown in Figure 3, the optimum incubation

temperature was 37°C for all five ECM proteins. In general, S735-binding to the collagens was significantly ($p<0.0001$) more temperature-dependent than for the two fibronectins. Similar results were obtained with the non-encapsulated strain B218 (result not shown).

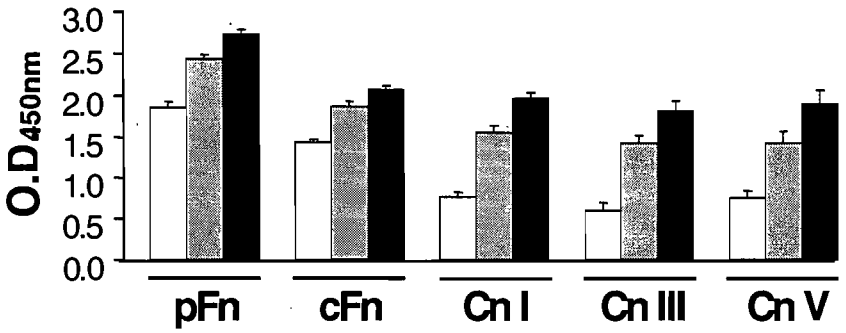

Fig. 3. Effect of incubation temperature on *S. suis* serotype 2 strain S735-binding to different ECM proteins. Wells were coated with either 10 μg/ml of plasma fibronectin (pFn), 25 μg/ml of cellular fibronectin (cFn), or 12.5 μg/ml of collagen type I (Cn I), type III (Cn III), or type V (Cn V) and were incubated with bacteria (10^8 C.F.U ml^{-1}) at different temperatures for 2 h. [4°C (white bars), 20°C (grey bars), and 37°C (black bars)]. Data are expressed as means ± standard deviations from at least three experiments performed in triplicate.

Binding capacity of various *S. suis* serotype 2 field strains to immobilized ECM proteins

All the strains tested (Table 2), bound to both fibronectins and collagen types I, III, and V. However, individual differences in the degree of binding of the various strains were observed (data not shown). Unlike strain S735, some field strains were able to bind to fibrin, and laminin. None of the strains bound to collagen type IV, elastin, human fibrinogen, or porcine fibrinogen. No correlation was noted between the ability of the various strains to bind to the ECM proteins and their source (diseased or healthy animals), the host from which they had been recovered (swine or human) (Table 2A), or their geographical origin (European or North American) (data not shown).

The unencapsulated mutant strain B218 bound significantly ($p<0.0001$) more to both fibronectins, collagen type III, and collagen type V than the parental strain 31533. In addition, unlike 31533, B218 bound to vitronectin and laminin (Table 2B).

	Diseased Pigs	Healthy Pigs	Humans	31533	B218
pFn	12/12	3/3	7/7	2.48±0.21*	3.16±0.07*
cFn	12/12	3/3	7/7	2.06±0.09*	2.34±0.12*
Cn I	12/12	3/3	7/7	1.83±0.10	1.81± 0.07
Cn III	12/12	3/3	7/7	2.34±0.12*	3.79±0.19*
Cn IV	0/12	0/3	0/7	0.07±0.04	0.25±0.02
Cn V	12/12	3/3	7/7	1.72±0.18*	3.89±0.07*
Fibrin	5/12	2/3	1/7	0.15±0.05	0.29±0.01
pFg	0/12	0/3	0/7	0.14±0.01	0.32±0.01
hFg	0/12	0/3	0/7	0.12±0.03	0.32±0.04
Lam	3/12	1/3	1/7	0.07±0.01*	1.32±0.19*
Vitro	0/12	0/3	0/7	0.10±0.01*	0.58±0.01*
Elast	0/12	0/3	0/7	0.13±0.06	0.28±0.03
Casein	0/12	0/3	0/7	0.19±0.02	0.25±0.09

Table 2. Adherence to ECM proteins of different *S. suis* serotype 2 field strains according to species of isolation or presence of disease (A). Effect of the capsule on adhesion between *S. suis* serotype 2 and ECM proteins (B)
B218: unencapsulated mutant derived from *S. suis* serotype 2 strain 31533. pFn, plasma fibronectin; cFn, cellular fibronectin; Cn I, collagen type I; Cn III, collagen type III; Cn IV, collagen type IV; Cn V, collagen type V; pFg, porcine fibrinogen; hFg, human fibrinogen; Lam, laminin; Vitro, vitronectin; Elast, elastin. Casein was used as negative control. Table 2B data are represented as means ($O.D._{450}$) ± standard deviations. *General linear model, ($p<0.0001$).

Proteolytic, formaldehyde, and heat treatments

Strain S735-binding to ECM proteins was affected by both proteolytic and heat treatments, decreasing by almost 90% of adhesion for all five ECM proteins tested (plasma and cellular fibronectin, and collagen types I, III and V). The 0.2% formaldehyde treatment did not affect binding to ECM proteins ($p<0.0001$) (results not shown). Similar results were obtained with the non-encapsulated strain B218 as well as with three different field strains randomly tested (results not shown).

Inhibition assays

Plasma fibronectin and collagen types I, III, and V, but not cellular fibronectin (even at high concentrations), competed with the homologous protein used in the ELISA assay, confirming the specificity of the binding (data not shown). Collagen at a concentration of 150 μg ml^{-1} inhibited binding not only to the homologous collagen but also to the two other collagens (data not shown). Preincubation of the bacteria with 150 μg ml^{-1} of plasma fibronectin significantly ($p<0.0001$) reduced binding to immobilized cellular fibronectin and collagen types I, III, and V (Fig 4). Since fibronectin is a multifunctional protein that also carries recruitment domains for host factors such as collagen and fibrin, strain S735-binding to collagen type IV and fibrin after preincubation with plasma fibronectin was evaluated. The addition of soluble plasma fibronectin promoted binding of this strain to collagen type IV ($p<0.0001$) (Fig. 4) but not to fibrin (data not shown).

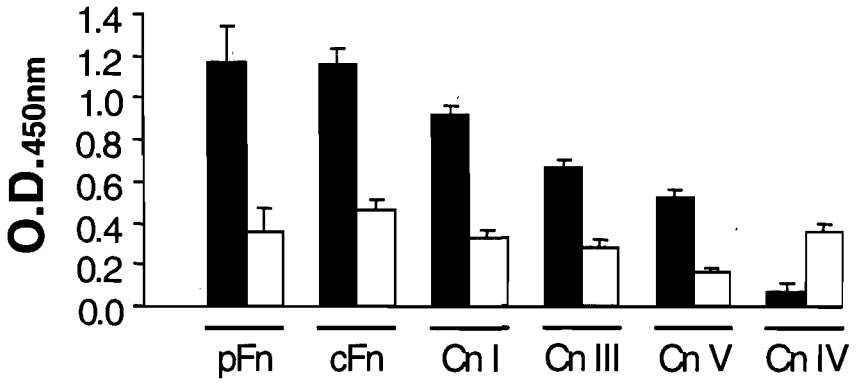

Fig. 4. Effect of pre-incubating bacteria for 90 min with 150 μg/ml of soluble plasma fibronectin on *S. suis* serotype 2 strain S735-binding to plasma fibronectin (pFn), cellular fibronectin (cFn), collagen type I (Cn I), collagen type III (Cn III), collagen type V (Cn V), and collagen type IV (Cn IV). Data represent the binding of *S. suis* preincubated with plasma fibronectin (white bars) or PBS (black bars). Bars represent means ± standard deviations of at least three experiments performed in triplicate.

DISCUSSION

Many adhesins have been described for *S. suis* serotype 2 in the last decade, including an 18-kDa galactosyl-alpha 1-4 galactose binding adhesin, which is present on different *S. suis* serotypes; a 60-kDa IgG-binding protein, which reacts with a large number of IgGs; and a 39-kDa glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which binds to various host proteins, including plasminogen and albumin (18, 27, 32, 38). GAPDH is known to be involved in the binding of *S. suis* to host tissues because mutants defective in the surface expression of this protein bind to a lesser degree to tracheal cells and porcine tracheal rings (6, 7). Lastly, a 64-kDa fibronectin-fibrinogen-binding protein (FBPS) with a high binding capacity for fibronectin and fibrinogen has recently been described (11). While the *fbps* gene is present in most *S suis* serotypes and pathotypes and while FBPS has been cloned and its binding activity verified (11), the expression of FBPS in the different serotypes and phenotypes was not studied, and no direct studies of whole *S. suis* bacteria or constructed *fbps* knockout mutant binding to fibronectin/fibrinogen have been carried out. The role of FBPS in bacterial pathogenesis is not well understood. However, studies with the *fbps* mutant suggest that this protein plays a role in *S. suis* colonization of various organs (11). We report here for the first time that whole *S. suis* cells are able to bind to fibronectin, confirming published data using purified proteins (11, 32). However, we could not confirm that *S. suis* binds to fibrinogen, probably because we used whole bacteria rather than purified protein.

Fibronectin exists in two main forms. Cellular fibronectin, which is an insoluble glycoprotein dimer that serves as a linker in the ECM, is produced by fibroblasts, chondrocytes, endothelial cells, macrophages, and certain epithelial cells. Plasma fibronectin, which is a soluble disulfide-linked dimer, is produced by hepatocytes and released in an unbound form into the plasma. Our study shows that all the *S. suis* serotype 2 strains tested are able to bind to both plasma and cellular fibronectin. In addition, *S. suis* serotype 2 binds to immobilized cellular fibronectin but not to soluble cellular fibronectin. Similar results have been obtained with Group B streptococci (GBS) and plasma fibronectin. In fact, GBS are able to bind to immobilized but not soluble plasma fibronectin (36). These results might be due to structural changes resulting in the exposure of determinants required for bacterial binding when fibronectin is attached to a solid surface. On the other hand, *S. suis* binds to both immobilized and soluble plasma fibronectin, probably due to the soluble disulphide-linked dimer conformation of plasma fibronectin, which allows a monomer conformation that exposes the determinants required for *S. suis* binding. Fibronectin appears to be one of the components mediating binding to and, in some cases, invasion of various host cell types by streptococci and staphylococci (3, 34, 40), but its role in *S. suis* binding to and/or invasion of epithelial, endothelial, and phagocytic cells (4, 9, 22, 30, 31, 39) remains to be elucidated.

Collagen is a major constituent of the ECM and, as such, may be a major target for pathogenic bacteria. Collagen is found in basement membranes (type IV), bone, skin, cartilage, tendons, and joints (12). This study demonstrated that *S. suis* serotype 2 is able to directly bind to collagen types I, III, and V, but not to type IV, probably due to structural differences (8) that might hide receptors required for *S. suis* binding. Interestingly, while *S. suis* is not able to bind directly to collagen type IV, it has the potential to adhere to this protein via a surface-bound fibronectin mechanism. Fibronectin-mediated collagen recruitment might represent a novel mechanism of colonization and immune evasion for *Streptococcus pyogenes* (12).

S. suis serotype 2 binds to both fibronectin and collagen in a bacterial and protein concentration-dependent manner. No binding was detected at bacterial concentrations under 10^7 bacteria ml^{-1}. Interestingly, it has been previously demonstrated *in vivo* that the presence of clinical signs and symptoms in diseased animals infected with *S. suis* correlates with high levels of bacteria in the bloodstream (5)..

Cross-inhibition was observed with soluble fibronectin and collagen in the ECM binding assay, indicating that fibronectin and collagen binding domains exist on the same or nearby bacterial surface adhesins. Similar results were obtained with GBS by Tamura and Rubens (37).

The bacterial ligand(s) that allows binding to ECM proteins could be either a protein (10) or a cell wall component (25). The dramatic reduction in binding by trypsin-, pronase-, and proteinase K-treated bacteria indicates, in the case of *S. suis*, a protein-mediated binding mechanism. This conclusion is supported by the fact that heat treatments (60°C and 100°C) also decreased binding to ECM proteins.

Most of the *S. suis* serotype 2 field strains tested behaved in a fashion similar to that of the reference strain. However, some were also able to bind to laminin, vitronectin, and fibrin, suggesting that they possessed other adhesins. On the other hand, interference by the CPS of some strains cannot be ruled out. In fact, our study showed that unencapsulated mutant B218 not only bound better to both fibronectins and collagen types III and type V than the encapsulated parental strain 31533, but it also bound to vitronectin and laminin, unlike strain 31533. In addition, Tikkanen *et al.* (1996) (38) reported an inverse relationship between the binding activity of the galactosyl-alpha 1-4 galactose binding adhesin and the expression of capsular polysaccharide. It has already been reported that CPS also influences the binding of *S. pyogenes, Staphylococcus aureus* (40), and *Lactobacillus acidophilus* (23) to some ECM proteins.

In summary, this study shows that *S. suis* serotype 2 is able to specifically bind to major constituents of ECM such as fibronectin and collagens, and that the adhesin(s) responsible for these

interactions are proteinaceous in nature. The identification of the *S. suis* surface protein(s) responsible for this binding phenotype and the creation of isogenic knock-outmutants of the associated gene(s) may give some insights about the role of these adhesins in the pathogenesis of the infection caused by *S. suis*. These efforts are ongoing in our laboratory.

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ARTICLE II

Isolation and characterization of α-enolase, a new fibronectin-binding protein from *Streptococcus suis*

Miriam Esgleas[1], Yuanyi Li[1], Mark A. Hancock[2], Josée Harel[1], J. Daniel Dubreuil[1] and Marcelo Gottschalk[1]*

[1]Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP) and Centre de Recherche en Infectiologie Porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, Québec, Canada. [2]Sheldon Biotechnology Centre, McGill University, Montreal, Québec, Canada.

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SUMMARY

Streptococcus suis is an important swine and human pathogen that causes meningitis, endocarditis, arthritis and septicaemia. As a zoonotic agent, *S. suis* also causes similar diseases in humans. Binding of pathogenic bacteria to extracellular matrix components enhances their adhesion to and invasion of host cells. In the present study we isolated and identified a novel fibronectin-binding protein from *S. suis*. The native protein (designated SsEno) possessed not only high homology with other bacterial enolases but also enolase activity. We cloned, expressed and purified the SsEno and showed that it is ubiquitously expressed by all *S. suis* serotypes and we identified its surface localisation using immunoelectron microscopy. ELISA assay demonstrated that SsEno specifically binds to fibronectin and plasminogen in a lysine-dependent manner. Additional Surface Plasmon Resonance assays demonstrated that SsEno binds to fibronectin or plasminogen with low nanomolar affinity. Inhibition experiments with anti-SsEno antibodies also showed that bacterial SsEno is important for the adhesion to and invasion of brain microvascular endothelial cells by *S. suis*. Overall, the present work is the first study to demonstrate a fibronectin-binding activity of a bacterial enolase and that, similar to other bacterial fibronectin-binding proteins, SsEno may contribute to the virulence of *S. suis*.

INTRODUCTION

Streptococcus suis is a major swine pathogen that causes septicaemia, meningitis, endocarditis, and arthritis (19). Of the 35 known serotypes, serotype 2 is most frequently isolated and associated with disease (19). It has been proposed that two serotypes (serotypes 32 and 34) be excluded from *S. suis* species and re-designated as *Streptococcus orisratti* (20). *S. suis*, especially serotype 2, has also been described as an important zoonotic agent that affects people in close contact with infected pigs or pork-derived products (37). Indeed, an important number of cases of human disease with a high rate of mortality in China were directly linked to a concurrent outbreak of *S. suis* infection in pigs (71).

Little is known about *S. suis* virulence factors. The capsule polysaccharide (CPS) is a critical virulence factor given that unencapsulated isogenic mutants are completely avirulent and rapidly cleared from the circulation in pig and mouse infection models (13, 58). However, non-virulent strains are also encapsulated, indicating that virulence of this pathogen is a multifactorial process (18). Other potential virulence factor have also been described in *S. suis,* including a haemolysin (suilysin), a 136-kDa muramidase-released protein (MRP), a 110-kDa extracellular factor (EF) protein, a hyaluronidase, a superoxide dismutase, various proteases, a serum opacity factor and different adhesins (3, 55).

The pathogenesis of *S. suis* infection is not fully understood and likely involves many steps (18). The binding between bacterial adhesins and host proteins is an essential step in the colonization of mucosal surfaces and it has also been implicated in bacterial invasion of host cells (60). Indeed, *S. suis* is able to bind to and, in some cases, invade endothelial and epithelial cells of human and porcine origin (4, 18, 33, 45, 66). However, the specific mechanisms involved in these interactions are unknown.

Some pathogens use host extracellular matrix proteins to potentiate their virulence. The extracellular matrix (ECM) is a stable macromolecular structure underlying epithelial and endothelial cells and surrounding connective tissue cells (69). Its composition varies among different organs, but the main components are fibronectin, collagen, elastin, laminin and glycosaminoglycans (32). Many of these proteins can potentially serve as surface receptors for bacterial binding to host cells via their adhesins (25, 54, 69). *S. suis* is able to adhere to fibronectin and different types of collagens (17). In fact, a 64-kDa fibronectin-and fibrinogen-binding protein (FBPS) with binding capacity for these two host proteins has been described for *S. suis* (14). The

role of FBPS in bacterial pathogenesis is not well understood. However, studies with the *fbps* mutant suggest that this protein could play a role in *S. suis* colonization of various organs (14).

Another *S. suis* adhesin that is able to bind to different host proteins such as plasminogen and albumin is the 39-kDa glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (23, 51). Evidence that this protein is involved in the binding of *S. suis* to host tissues is provided by findings that mutants deficient in the surface expression of GAPDH have decreased binding to tracheal cells and porcine tracheal rings (9, 10). GAPDH is also a cytosolic enzyme that participates in the degradation of carbohydrates via glycolysis and other catabolic pathways as well as in glucose synthesis via gluconeogenesis. Another enzyme essential for glycolysis and gluconeogenesis is enolase (39). This cytosolic enzyme catalyses the conversion of 2-phosphoglycerate (2-PG) into phosphoenolpyruvate (PEP) via the elimination of a water molecule (70). Enolase has also been identified as a novel plasminogen receptor on the surface of many eukaryotic cells (40, 43). This unexpected feature has also been described in several bacteria (such as *Staphylococcus aureus* and many streptococci), fungi and nematodes (5, 26, 27, 41, 48, 57). While the role of plasminogen as an important component in streptoccoci adhesion is well established (49), the role of enolase in bacterial adherence and host cell invasion has not been previously investigated.

In addition to identifying and inducing the expression of *S. suis* α-enolase (SsEno), we now demonstrate for the first time that this protein not only has plasminogen-binding activity but also has important fibronectin-binding properties. Finally, we also demonstrate that surface SsEno, which is present in all the described serotypes of *S. suis*, may play an important role in the adhesion and invasion process of host endothelial cells.

METHODS

Bacterial strains and growth conditions

Reference strains from the 35 different *S. suis* serotypes came from our collection. Highly virulent strain 166' of *S. suis* serotype 2 (8) was kindly provided by Dr. M. Kobisch, AFSSA, Ploufragan, France. *S. suis* strains were grown in Todd Hewitt broth (THB) (BD, Sparks, MD) as previously described (1). Late exponential-phase bacteria were washed in phosphate-buffered saline (PBS) (pH 7.3). *Escherichia coli* strain BL21DE3 was used for expression experiments. *E. coli* was grown in Luria-Bertani broth or agar plates. Ampicillin (100 $\mu g\ ml^{-1}$) (Roche, Indianapolis, IN) was used in growth media when required.

Isolation of 52-kDa protein by fibronectin-affinity chromatography

Strain 166' was harvested from THB by centrifugation at 5500 × *g* for 15 min, washed twice in phosphate buffered saline (PBS, 0.01 M, pH 7.4), and re-suspended in HEPES 10 mM, pH 7.4. Bacteria were disrupted by sonicating the bacterial suspension with an ultrasonic probe on ice for 30 minutes with 15-second intervals of cooling on ice at 80% duty cycle (Sonics & Materials, New Town, Connecticut, USA). Cell debris and non-lysed bacteria were removed by centrifugation. Supernatant proteins were concentrated to a volume of <1.0 ml using Amicon Ultra-15 concentrators (Millipore, Carringtwohill, Cork, Ireland) and applied to a 5 ml fibronectin-coupled CNBr-activated Sepharose 4B column (Amersham Biosciences AB, Uppsala, Sweden) pre-equilibrated with 0.1 M Tris/HCl buffer, pH 8.0. After washing with 5-column volumes of this buffer, bound proteins were eluted with 30 ml of 8 M urea. Starting lysates and fractionated samples were analyzed by SDS-PAGE (12.5% polyacrylamide) under reducing conditions, followed by Coomassie Blue staining. Protein concentrations were determined by the Lowry method (36). Samples were maintained at -70°C until analysis.

N-terminal sequencing was carried out with chromatographic fractions containing fibronectin-binding proteins after being electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore) and visualised by staining with 0.1% Ponceau S (Sigma-Aldrich, St. Louis, Mo) in 1% acetic acid. Bands of interest were excised, destained with distilled water, and subjected to peptide sequencing at the Biotechnology Research Institute (Montreal, Canada).

Cloning, expression and purification of recombinant SsEno (rSsEno)

The peptide sequence identified by N-terminal sequencing of the predominant fibronectin-binding protein was subjected to a homology search using the BLAST program against the strain P 1/7 genome sequence of *S. suis* (http://www.sanger.ac.uk/Projects/S_suis/). Database search revealed 100% homology to the N-terminal region of a 1308 bp open reading frame (ORF) that

corresponds to the *S. suis* enolase (SsEno). The sequence was used to design primers for PCR amplification of the SsEno gene. The primers used were the Eno1-forward primer 5'-TATAA**GGATCC**TTGTCAATTATTACTGATGTTTACGC-3', introducing a *BamHI* site (boldface and underline letters) and the Eno2-reverse primer 5'-TATA**AAGCTT**TTATTTTTTCAAGTTGTAGAATGAGTTCAAGCC-3', introducing a *HindIII* site (boldface and underline letters). Automated sequencing was then used to check the amplified enolase gene. The verified complete gene was cloned into pET-32a (Novagen, Madison, WI,) using the created *BamHI* and *HindIII* sites. This plasmid contains a His-tag-encoding sequence of about 25 kDa. The plasmid pET-32a-SsEno was introduced into *E. coli* Bl21DE3 for IPTG-inducible expression of recombinant SsEno. Under native conditions, the His-tagged fusion protein was purified by HisTrap chromatography according to the manufacturer's protocols (Amersham Biosciences). Protein-containing fractions were determined by SDS-PAGE (12.5%) followed by Coomasie blue staining. Purified rSsEno was used to produce polyclonal antibodies to SsEno in rabbits as previously described (34). To prepare affinity purified anti-SsEno IgG, the polyclonal serum was subjected to purification on protein-A Sepharose CL-4B (Amersham Pharmacia) (48).

Enolase Activity

α-Enolase activity was determined by measuring the transformation of $NADH{\cdot}H^+$ to NAD^+ as described previously (48) with some modifications. Briefly, the enzymatic reactions were performed at 37°C in 100 mM HEPES buffer, pH 7.0, containing 5.0 mM $MgSO_4$, 0.2 mM NADH (Roche), 0.25 mM 2-phosphoglycerate (2-PGE) (Sigma), 1.2 mM ADP (Roche), 10.7 IU lactate dehydrogenase (Roche) and 2.5 IU pyruvate kinase (Roche) in a final volume of 1 ml. The reaction was started by adding 100 µl of the test solution containing rSsEno (from 0 to 20 µg). The α-enolase activity was measured in terms of the rate of reduction in the absorbance at 340 nm (*i.e.* increase in the production of NAD from $NADH{\cdot}H^+$). Rabbit muscle Enolase (Sigma) was used as a positive control and buffer without enolase was used as a negative control.

Localisation of SsEno in *S. suis*

To evaluate the presence of SsEno in different bacterial compartments, *S. suis* strain 166' was grown overnight at 37°C with agitation in 30 ml of THB. The subcellular components (supernatant, cell wall, cytoplasmic and membrane fractions) were fractionated with mutanolysin by using the method of Yother and White (72). Equivalent amounts of all fractions were analysed by SDS-PAGE (12.5 % polyacrylamide) and Western blot using optimally diluted rabbit anti-SsEno IgG.

The surface exposure of SsEno was examined by using immunelectron microscopy. Briefly, *S. suis* 166' was grown in 5 ml of THB until late exponential phase, centrifuged, and resuspended in PBS (pH 8.0). A 20 µl aliquot of the bacterial suspension was placed on nickel-Formvar grids (INRS, Institut Armand Frappier, Laval, Canada) and allowed to partially air dry. After blocking for 30 min with 10% normal donkey serum in dilution buffer (PBS-1% bovine serum albumin-1% Tween 20, pH 8.0), the grids were incubated with optimally diluted rabbit anti-SsEno IgG for 2 h at room temperature. The grids were washed three times with PBS, pH 8.0, 1% Tween 20 and incubated for 1 h at room temperature with 12-nm colloidal gold-Affinipure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). After several washes with PBS-1% Tween 20, bacteria were stained with 1% phosphotungstic acid and examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV. Bacteria coated with normal rabbit antibodies served as a negative control.

rSsEno Binding Assays for Plasminogen and Fibronectin

Purified rSsEno binding to immobilized fibronectin (Roche) and plasminogen (Sigma) was first determined by ELISA. Maxisorp® flat-bottom microtiter 96-well plates (Nunc, VWR, Mississauga, ON, Canada) were coated with 100 µl of serial dilutions of fibronectin or plasminogen, depending on the experiment, in 0.1 M carbonate coating buffer (0.15% [wt/vol] Na_2CO_3, 0.1% [wt/vol] $MgCl_2$ - $6H_20$, 0.3% [wt/vol] $NaHCO_3$ [pH 9.6]) and incubated overnight at 4°C. Casein-coated wells served as a control for non-specific adhesion of rSsEno. The plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST, pH 7.3), and 200 µl of 3% (w/v) non-fat dry milk in PBST was added to each well to prevent non-specific binding. After 1 h at 37°C, the wells were washed three times with PBST. Next, 100 µl of 5 µg ml^{-1} of rSsEno was added and the plates were incubated for 2 h at 37°C. After several washes with PBST, optimally diluted rabbit anti-SsEno IgG were added to each well and the plates were incubated for 1h at 37°C. The wells were washed three times with PBST and specific horseradish peroxidase-labelled IgG (Jackson Immunoresearch Laboratories) was added. Plates were then incubated for 1 h at 37°C with the secondary antibody and 3,3',5,5'-tetramethylbenzidine (Zymed, San Francisco, CA, USA) was used as the enzyme substrate according to the manufacturer's instructions. The reactions were stopped by adding 25 µl of H_2SO_4 (1N) and were read at 450 nm using a microplate reader (Uvmax; Molecular Devices, Menlo Park, CA, USA).

Binding interactions between rSsEno (75 kDa) and fibronectin (440 kDa) or plasminogen (92 kDa) were also analyzed in real-time using Biacore 2000/3000 instrumentation (BiacoreAB, Uppsala, Sweden). Surface Plasmon Resonance (SPR) assays were performed on research-grade CM4 sensor chips at 25°C using filtered (0.2 µm) and degassed HBS-EP running buffer (10 mM

HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween 20). Prior to all experiments, the purity of protein preparations (>95%) was assessed by SDS-PAGE (12.5% polyacrylamide) under non-reducing and reducing (5% (v/v) 2-mercaptoethanol) conditions, followed by Coomassie Blue and silver staining. Protein concentrations were determined using the Lowry method (36). The zwitterionic detergent Empigen was from Calbiochem; all other chemicals were of reagent-grade quality.

Fibronectin or plasminogen (10 μg ml^{-1} in 10 mM sodium acetate pH 4.5) was immobilized to sensor chip surfaces using the Amine Coupling Kit (BiacoreAB) according to the manufacturer's recommendations. Similarly, corresponding reference surfaces were prepared in the absence of ligand addition. rSsEno (0 –250 nM diluted in HBS-EP) was injected in-tandem over reference (no ligand) and active (290 relative units (RU) fibronectin or 140 RU plasminogen-immobilized) surfaces at 25 μL min^{-1} (600 sec association + 600 sec dissociation). Surfaces were regenerated at 50 μL min^{-1} using two 30-second pulses of solution I (HBS-EP containing 1 M NaCl, 5 mM NaOH, and 0.03% (v/v) Empigen) and solution II (HBS-EP), followed by EXTRACLEAN and RINSE procedures. Additional assay controls to test for binding specificity (BSA negative control), surface performance (consistent replicate injections), and lack of mass transport were performed as recommended by the manufacturer.

All binding data were doubled-referenced (42) and representative of triplicate injections acquired from three independent trials. Due to the slow off-rates observed (less than 10% dissociation over 10 minutes), it was not possible to evaluate the data using standard kinetic (*ka*, *kd*) analysis in the BIAevaluation 4.1 software (BiacoreAB). Alternatively, steady-state binding responses were averaged (540-580 sec plateau) and then analyzed globally as a function of rSsEno concentration. For non-linear regression analysis (R_{eq} vs. C), overall affinity constants were derived by fitting the data to a steady-state binding model: $R_{eq} = (R_{max} \bullet C) / (K_D + C)$ where R_{eq} is the binding response at equilibrium, R_{max} is the maximal specific binding to the ligand surface, C is the concentration of analyte in solution, and K_D is the equilibrium dissociation constant. For linear regression analysis, K_D values were derived as the negative inverse of the slope generated by Scatchard transformations (R_{eq}/C vs. R_{eq}).

Lysine-dependent rSsEno binding to fibronectin and plasminogen

To address the importance of lysine residues in the binding of rSsEno with fibronectin, competition binding assays were performed using the lysine analog ε-amino-n-caproic acid (EACA). Briefly, fibronectin and the positive control plasminogen (10 and 1 μg ml^{-1} respectively) were incubated overnight at 4°C in Maxisorp® flat-bottom microtiter 96-well plates and an ELISA was performed as described above using 20 μg ml^{-1} of rSsEno mixed with different concentrations

of EACA (1, 5, 10 and 50 mM, Sigma) and rabbit anti-SsEno IgG as a primary antibody. Binding of SsEno to fibronectin and plasminogen in the absence of EACA competitor was considered as 100%.

Role of SsEno in adhesion and invasion of *S. suis* to PBMEC

The porcine brain microvascular endothelial cell (PBMEC) line PBMEC/C1-2 (62), already tested with *S. suis* in our laboratory (66) was used. The adhesion assay to quantify total cell-associated (intracellular plus surface-adhered) bacteria was performed as previously described (44, 66), with some modifications. Briefly, *S. suis* strain 166' (10^7 CFU ml^{-1}) was incubated with equal concentrations of either normal rabbit IgG or purifed rabbit anti-SsEno IgG for 1 h at 37°C in fresh cell culture medium without antibiotics. Confluent cell monolayers were inoculated with 1 ml aliquots of either bacterial suspension. The plates were centrifuged at 800 x *g* for 10 minutes and incubated for 2 h at 37°C with 5% CO_2. The monolayers were then vigorously washed 5 times to eliminate non-specific bacterial attachment, incubated for 10 min at 37°C in the presence of 200 µl of 0.05% trypsin-0.03% EDTA, and disrupted by scraping the bottom of the well and by repeated pipetting to liberate cell-associated bacteria in the presence of ice-cold deionized water. Serial dilutions of this cell lysate were plated onto THB agar and incubated overnight at 37°C, after which the bacteria were counted. The invasion assay to quantify intracellular bacteria was performed in a similar manner. However, to kill extracellular and surface-adhered bacteria after the initial infection period, cells were washed twice with PBS, and 1 ml of cell culture medium containing 100 µg ml^{-1} of gentamicin and 5 µg ml^{-1} of penicillin G (Sigma) was added to each well. After incubation for 1 h at 37°C with 5% CO_2, monolayers were washed 3 times with PBS and processed as described above. Results obtained with bacteria incubated with normal rabbit IgG were considered as 100% of adhesion or invasion.

Statistical analysis

All experiments were performed at least three times with samples in triplicate. All numerical data presented here are expressed as means ± standard deviation. Statistical significance was determined using Student's *t* test. Differences were considered significant at $P \leq 0.05$ (*).

RESULTS

Identification of a major *S. suis* fibronectin-binding protein of 52-kDa as an α-enolase

Loading of *S. suis* lysates onto a fibronectin-affinity column resulted in the specific retention and elution of a predominant native 52-kDa protein along with very few faint bands of additional proteins (Fig. 1). N-terminal amino acid sequencing of the 52 kDa protein revealed the following 14 residues: MIITDVYAREVLDS. Comparison of this 14-amino acid N-terminal sequence with the genomic sequence database of *S. suis* (http://www.sanger.ac.uk/Projects/S_suis/) revealed 100% homology to the N-terminal region of a 1308 bp ORF, which encodes a putative 435-amino acid protein with a deduced molecular weight of 47.09 kDa. A protein BLAST search with the putative protein sequence in the NCBI sequence database (http://www.ncbi.nlm.nih.gov/BLAST/) revealed significant homology to bacterial α-enolases, a family of proteins involved in carbohydrate transport and metabolism. The identity between the putative *S. suis* protein and the α-enolase from the closely related streptococci is more than 93% (data not shown). These results indicated that this *S. suis* 52 kDa protein is most likely an α-enolase (SsEno). Sequence analysis showed that the SsEno contains neither a signal peptide nor an LPXTG motif. This protein also does not contain choline-binding repeats. Immunoblot analysis confirmed the cross-reactivity of SsEno with polyclonal antibodies against *Streptococcus pyogenes* enolase (SEN) (data not shown).

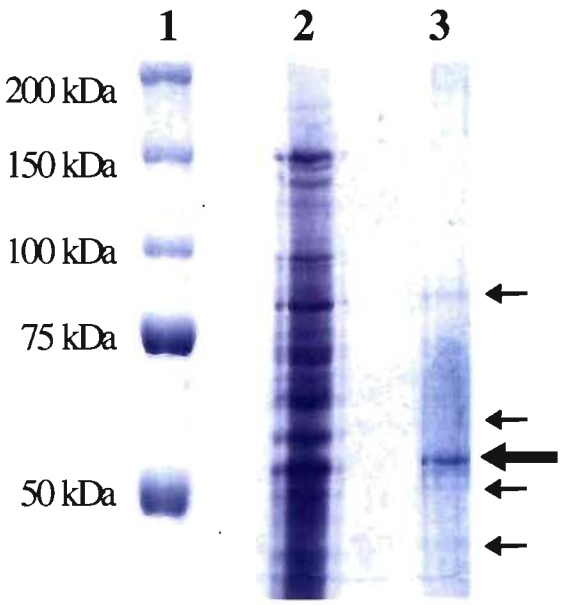

Fig. 1. Isolation of a 52-kDa protein by fibronectin-affinity chromatography. *S. suis* lysate was applied to a fibronectin coupled CNBr-activated Sepharose 4B column pre-equilibrated with 0.1 M Tris/HCl buffer, pH 8.0. After washing, bound protein was eluted with 30 ml of 8 M urea elution buffer. Elution profiles were analyzed by SDS-PAGE (12,5 % polyacrylamide) with Coomassie Blue staining. Lane 1: Molecular markers, lane 2: *S. suis* total proteins, lane 3: *S. suis* Fibronectin-binding proteins. Large arrow : 52 kDa enolase; small arrows: other fibronectin-binding proteins.

rSsEno has enolase activity

rSsEno was successfully cloned and expressed as a His-tagged fusion protein of ~75 kDa in *E. coli*. The higher molecular weight of the cloned protein compared to the native protein (52 kDa) is due to the N-terminal sequence of about 25 kDa containing the His-tag added by the plasmid. The recombinant protein was purified to homogeneity (>95%) using HisTrap affinity chromatography (Fig. 2) and it presents identical cross-reactivity to the native protein as shown by immunoblot with antibodies against SEN (results not shown).

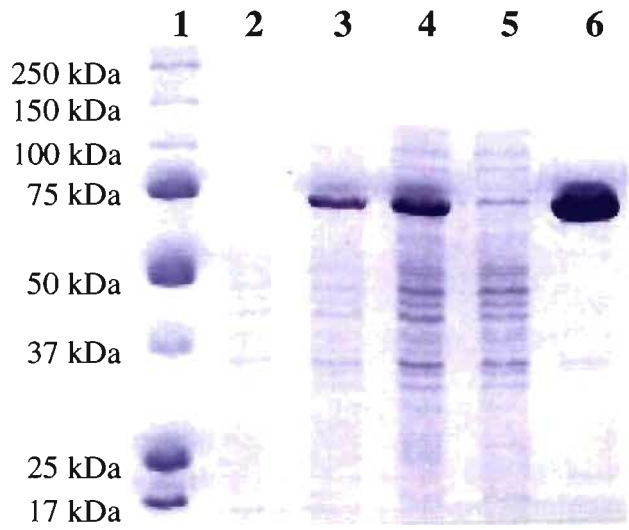

Fig. 2. Expression and purification of recombinant SsEno. *SsEno* gene was cloned into pET-32a which was introduced into *E. coli* Bl21DE3 for an IPTG-inducible expression of rSsEno (recombinant *S. suis* enolase). The His-tagged fusion protein was purified by chromatography on HisTrap column according to the manufacturer's protocols. Lane 1 represents molecular weight markers, lane 2: non induced *E. coli*, lane 3: induced *E. coli*, lane 4: French press control, lane 5: His-trap non adhered proteins, lane 6: His-Trap purified SsEno.

To confirm that the fibronectin-binding protein candidate is an α-enolase, its activity was assayed in a coupled enzyme assay for enolase. Similarly to the positive control used, we demonstrated that the recombinant protein was able to convert NADH to NAD resulting in a change in absorbance at 340 nm (Fig. 3). This indicated that pyruvate was converted to lactate and NADH by lactate dehydrogenase, confirming the conversion of phosphoglycerate to phosphoenolpyruvate by α-enolase and finally to pyruvate in the presence of externally provided pyruvate kinase and ADP in a sequential manner.

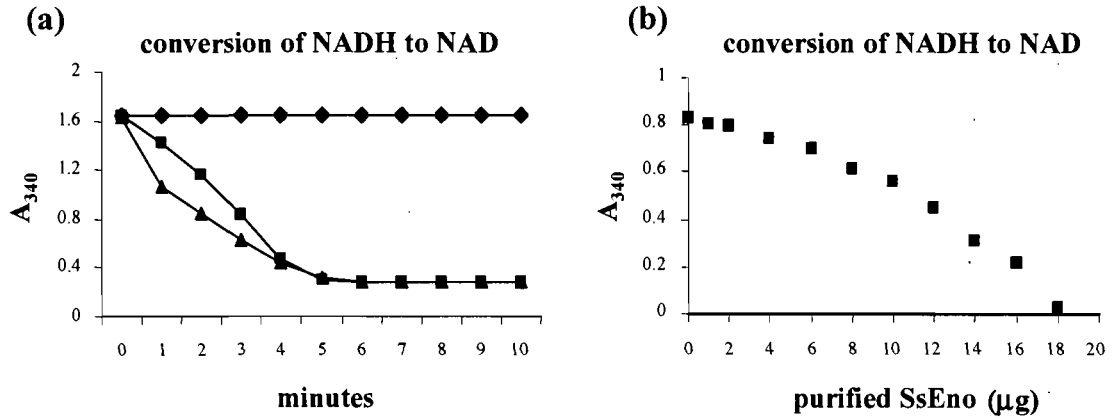

Fig. 3. Enolase activity assay for recombinant SsEno. Alpha-Enolase activity of the purified rSsEno was measured using a coupled enzyme assay catalyzing 2- phosphoglycerate to phosphoenolpyruvate as described under "Experimental Procedures" (a) Enzyme kinetics of the purified rSsEno was determined, using 1 µg of the purified rSsEno (▲) or 1 µg of muscle rabbit enolase as positive control (■) as the enzyme source. As negative control, enolase was omited (♦). Alpha-enolase activity of SsEno was also measured as described in A. (b) complementary assay in which different SsEno concentrations were used as enzyme source and the enzyme activity in the reaction mixture was measured at the end of 1 min incubation. Data represent results of one individual experiment. The enolase assay was done at least in triplicate.

SsEno has fibronectin- and plasminogen-binding activities

Prior to all ELISA and SPR analyses, the purity (>95%) of commercially-available fibronectin and plasminogen (data not shown) and rSsEno preparations was determined by scanning densitometry of the protein on an SDS-PAGE gel stained with Coomassie blue (data not shown) and with a silver-stained SDS-PAGE gel (Fig. 4a). Qualitatively, ELISA assays provided the initial demonstration that rSsEno specifically interacts with immobilized fibronectin and plasminogen (positive control) in a saturable, dose-dependent manner (Fig. 4b-c). Under similar assay conditions, rSsEno failed to interact with immobilized casein as a negative control (Fig. 4d). Competition experiments with the lysine analogue EACA showed lysine-dependent interactions between rSsEno and fibronectin or plasminogen (Fig. 5c). Notably, the inhibition of rSsEno binding by EACA was more pronounced with plasminogen as compared to fibronectin.

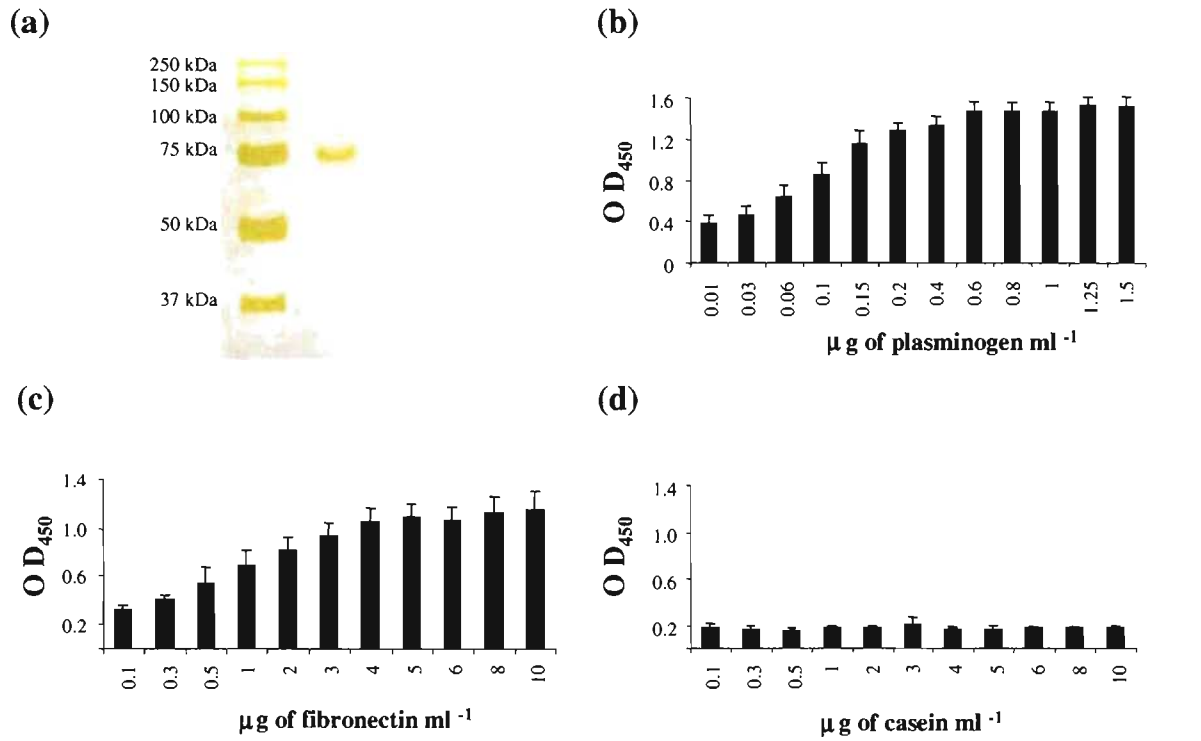

Fig. 4. Qualitative binding of SsEno to fibronectin and plasminogen. Silver nitrate staining (a) of SsEno used for ELISA and SPR tests. Representative ELISA analysis for the binding of SsEno (5 µg ml^{-1}) to immobilized plasminogen (b), fibronectin (c) and casein (d, negative control). Binding was evaluated after 2 h. Data are expressed as means ± standard deviation of at least three experiments with samples in triplicate.

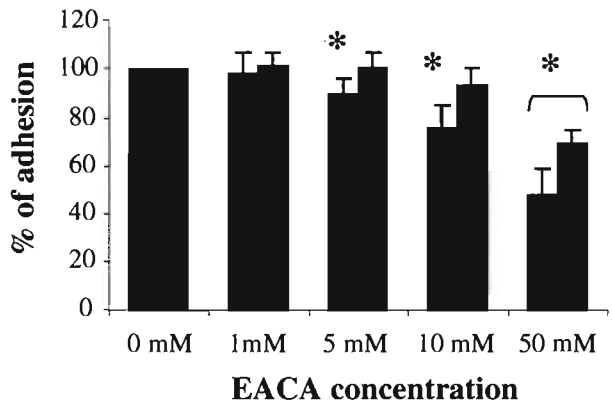

Fig. 5. Lysine-dependent binding of SsEno to fibronectin and plasminogen. Different EACA concentrations were used to evaluate the role of the lysines residues in the binding of SsEno to plasminogen (black bars) and fibronectin (grey bars) by ELISA as described in "Experimental procedures". An asterisk indicates that the *P* value is <0.05 for a comparison with the level of adhesion without competitor (considered as 100% of adhesion). Data are expressed as means ± standard deviation of at least three experiments with samples in triplicate.

To complement the ELISA data sets with more detailed quantitative kinetic analysis, rSsEno binding interactions were also examined in real-time by SPR. Specific, dose-dependent binding of rSsEno to immobilized fibronectin or plasminogen (positive control) was characterized by slow association (*ka*) and slow dissociation (*kd*) rates in both cases (Fig. 6a,c). Under similar assay conditions, bovine serum albumin failed to interact with the immobilized surfaces as a negative control (data not shown). Due to the slow off-rates observed for rSsEno binding to fibronectin or plasminogen (less than 10% dissociation over 10 minutes), it was not possible to evaluate the data using standard kinetic (*ka*, *kd*) analysis in the BIAevaluation 4.1 software (BiacoreAB). Alternatively, steady-state binding responses were averaged at the end of the association phase (540-580 sec plateau) and then analyzed globally as a function of rSsEno concentration. Linear and non-linear regression analyses (Fig. 6b,d) indicated similarly high-affinity interactions between SsEno-fibronectin (K_D ~21 nM) and SsEno-plasminogen (K_D ~14 nM).

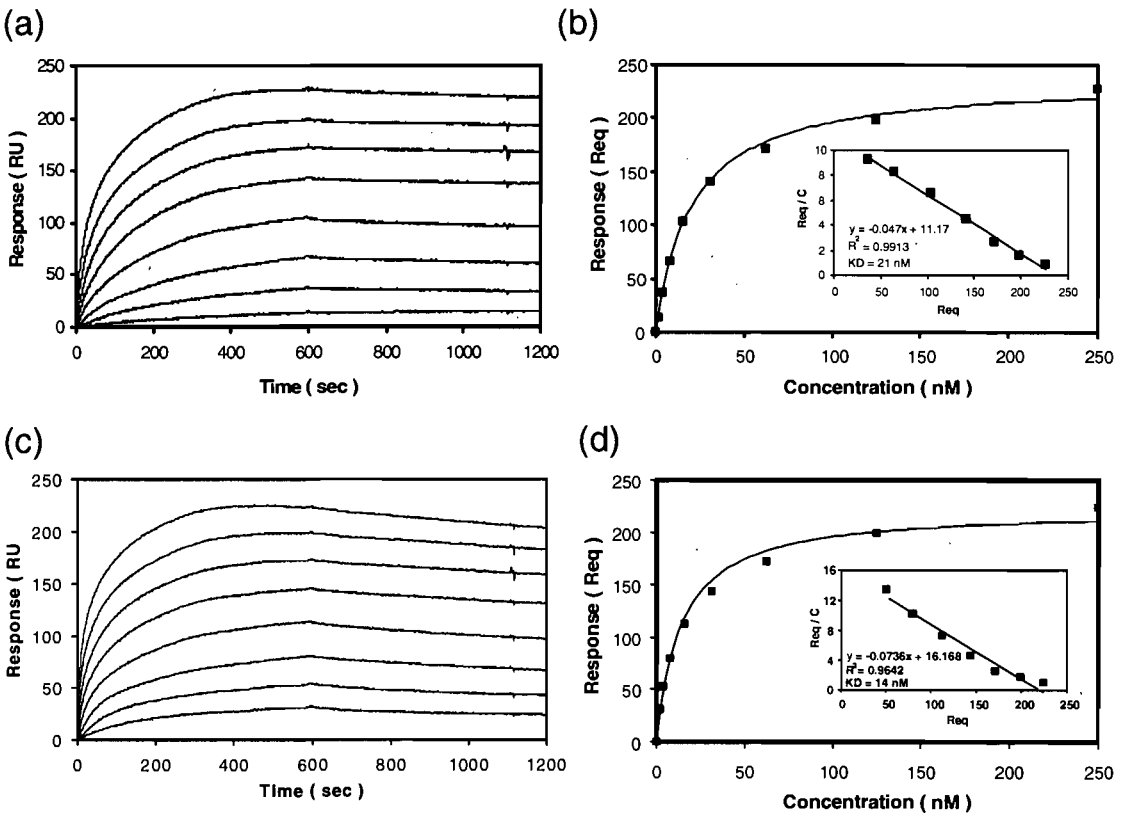

Fig. 6. Quantitative binding of SsEno to fibronectin and plasminogen. Representative SPR analysis of rSsEno (0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250 nM) injected (600 sec association + 600 sec dissociation) over amine-coupled fibronectin (290 RU; (a)) or plasminogen (140 RU; (c)) at 25 µL/min. Equilibrium binding responses (R_{eq}) were averaged (540-580 sec) and then evaluated according to the steady-state affinity model (R_{eq} vs C; fibronectin (b); plasminogen (d)). Subsequent scatchard transformations (R_{eq}/C vs R_{eq}; insets) indicate similar high-affinity interactions between SsEno-fibronectin (K_D ~21 nM) and SsEno-plasminogen (K_D ~14 nM).

SsEno is present both intracellularly and at the bacterial surface

Western blot results showed that SsEno is present in *S. suis* supernatant, cell wall, and cytoplasmic fractions, with negligible amounts in the membrane fraction (data not shown). Immunoelectron microscopy using the same antibody confirmed the surface location of SsEno on *S. suis* (strain 166') cells (Fig. 7a). In contrast, no labeling was observed for the negative control (Fig. 7b).

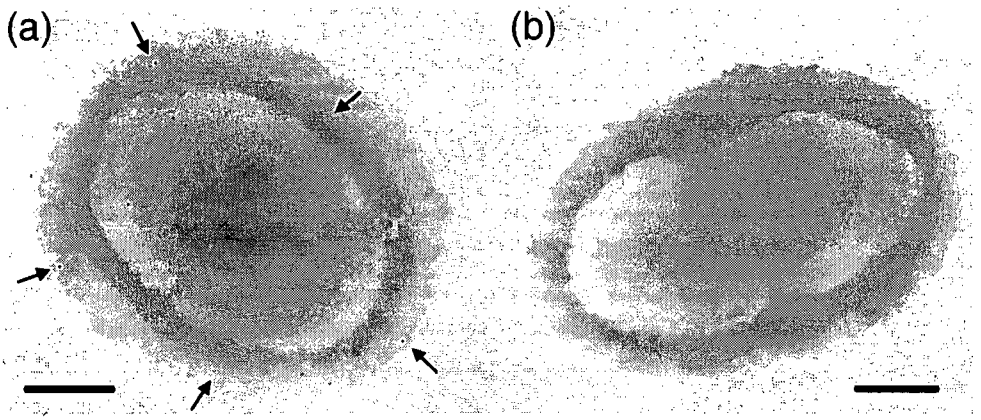

Fig. 7. Surface localization of SsEno on *S. suis*. (a) Immunogold electron microscopic detection of the location of SsEno (magnification × 45,000) on *S. suis* pretreated with rabbit anti-SsEno IgG, followed by colloidal gold-labeled anti-rabbit IgG (gold particle sizes are 12 nm; indicated by the arrow). (b) Normal rabbit IgG was used as a negative control. Bars, 250 nm.

SsEno participates in *S. suis* adhesion to and invasion of PBMEC

To evaluate the potential role of SsEno in *S. suis* adhesion to and invasion of PBMEC, bacterial SsEno was blocked with affinity purified rabbit anti-SsEno IgG. As shown in Fig. 8, anti-SsEno IgG treatment decreased to ~46% the adhesion of *S. suis* serotype 2 strain 166' to PBMEC compared with the control. The invasion of *S. suis* to PBMEC was also decreased to ~79%, compared with the control.

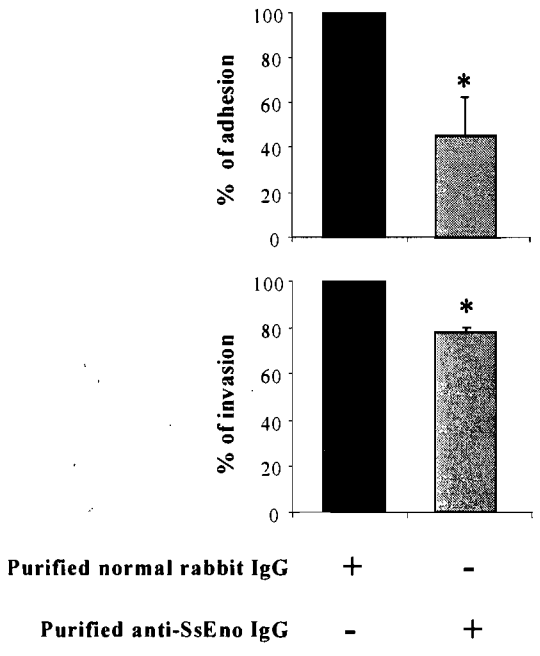

Fig. 8. Decrease of the adhesion and invasion of *S. suis* strain SS166 to PBMEC by antibodies against SsEno. *S. suis* strain 166' was preincubated with the appropriate concentrations (10 μg) of antibodies at 37°C for 60 min prior to infection. Adhesion was determined after exposure of PBMEC to *S. suis* (10^7 CFU ml^{-1}), followed by extensive washing of nonadherent bacteria and cell lysis to obtain *S. suis* viable plate counts on THB. Invasion was determined as described above, except that after washing the bacteria and cells were exposed to antibiotics to kill extracellular bacteria. An asterisk indicates that the *P* value is <0.05 for a comparison with the level of adhesion/invasion with control antibodies (considered as 100% adhesion/invasion).

DISCUSSION

Over the past 15 years, *S. suis* infections have emerged as a major problem for the swine industry worldwide, and they are also considered an important zoonotic agent (19, 61, 71). The pathogen is able to spread systemically from the nasopharynx, resulting either in general septicaemia or infections of specific organs (e.g. endocarditis, meningitis, arthritis), followed frequently by death (18, 38). The palatine and pharyngeal tonsils are both potential portals of entry for *S. suis*, although the mechanisms and virulence factors that enable the pathogen to disseminate throughout the animal and colonize different tissues are not well understood.

Adhesion of pathogens to host tissues is a critical early step in the process of infection. The ability to bind to fibronectin is a characteristic that has been reported for many pathogens (25). As fibronectin plays an important role in diverse normal physiological processes, its targeting appears to be an example of the exploitation of a host cell process in the establishment, maintenance or dissemination of infection (29). Although a previously reported fibronectin-and fibrinogen-binding protein (FBPS) has been described for *S. suis* (14), a *fbps* mutant was shown to adhere to fibronectin at similar levels as those presented by the wild-type parent strain (67), suggesting that other major adhesins are also involved.

In the present study, using fibronectin-coupled affinity chromatography, we have identified a predominant *S. suis* fibronectin-binding native protein of 52 kDa. Subsequently, the protein was identified as an α-enolase based upon to the high similarity of its sequence with other described bacterial enolases (93% with other streptococcal α-enolase) and our biochemical tests confirmed enolase activity. Furthermore, SsEno is also cross-reactive with anti-SEN antiserum, which has previously been shown to react with α-enolase-like molecules on streptococci (48). This is the first time that the fibronectin-binding activity of an α-enolase is described. These results were confirmed using rSsEno cloned and expressed in the present study. In fact, Antikainen *et al.* (2007) have recently reported that α-enolases from other gram positive bacteria such as *S. pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Lactobacillus crispatus* and *Lactobacillus johnsonii*, do not possess the ability to bind to fibronectin. Thus, the interaction of enolase with fibronectin may serve different purposes in *S. suis* than in the pathogenic and commensal bacteria studied in that work. Interestingly, Jing *et al.* (22) has recently proposed, after a complete proteome analysis, the enolase as one of two putative virulence-associated factors for *S. suis* serotype 2. However, the hypothetical plasminogen binding of the *S. suis* enolase (based on previous studies

performed with other streptococci) is proposed in that study as the mechanism used by this virulence factor to participate in the pathogenesis of the infection caused by this pathogen.

To possess fibronectin-binding activity, it was anticipated that SsEno should be exposed at the surface of bacteria. Fractionation of *S. suis* strain 166' showed that SsEno is present in all the subcellular components, including the bacterial surface, as previously demonstrated for other pathogens including *S. pyogenes* (48). ELISA tests carried out with whole bacteria and anti-SsEno also showed a clear recognition of this protein at the bacterial surface (M. Esgleas and M. Gottschalk unpublished observations). Electronic microscopy corroborated this surface localization and also showed that SsEno is present at the surface in low abundance, a feature that has also been described for *S. pneumoniae* enolase (30). Moreover, such a relatively low amount has proven sufficient for contributing to bacterial pathogenesis (7). Interestingly, in addition to serotype 2, all reference strains of other serotypes of *S. suis* were found to express surface-exposed SsEno (M. Esgleas and M. Gottschalk unpublished observations). The mechanisms by which this protein is translocated to the bacterial surface are still unknown. In fact, the amino acid sequence of SsEno shows the absence of an N-terminal signal sequence and of an anchor carboxyl terminus LPXTG motif. It has been demonstrated that this LPXTG motif is necessary for the translocation of proteins to the bacterial surface because it is recognized by a transpeptidase (sortase A) that cleaves between the Thr and Gly residues (53). Once cleaved, the carboxyl group of the Thr residue is linked to a free amino group of a branch peptide within the peptidoglycan cell wall permitting the anchorage of these LPXTG containing proteins to the bacterial cell wall (53). Interestingly, we have recently shown that disruption of srtA gene in *S. suis* results in only a slight decrease in adhesion to fibronectin (68), confirming that anchorless (such as the enolase) rather than LPXTG motif proteins play a major role in the adhesion to this extracellular matrix protein.

To date, the major function described for surface enolases is a strong plasminogen-binding activity (47, 48). In this study we confirmed that, in addition to a previously described GAPDH (23), SsEno is also a *S. suis* plasminogen-binding protein. Although it has recently been demonstrated that enolases of other gram-positive bacteria bind to laminin and/or collagen (2), we could not identify any adhesion activity of rSsEno to any of these two proteins (M. Esgleas and M. Gottschalk unpublished observations). Interestingly, in addition to the fibronectin and plasminogen-binding activities of the rSsEno, a heat-shock protein activity (as previously described for other enolases (21, 50)) and an IgG-binding properties of this protein have also been observed (M. Esgleas and M. Gottschalk unpublished observations).

The binding of purified rSsEno to both plasminogen and fibronectin was demonstrated qualitatively by ELISA and quantitatively by SPR. Interestingly, a higher amount of fibronectin, compared to that used for plasminogen, had to be used in the ELISA test to obtain similar absorbances. Although this might be interpreted as a significant higher affinity (which was not confirmed by SPR), this is not the case. The ELISA test was used in this study as a qualitative test, since plasminogen and fibronectin have different molecular weights and their attachment to plastic wells may vary with the conditions used for the ELISA. In particular, SPR analysis clearly indicated that rSsEno interactions with plasminogen and fibronectin were of similar high affinity (K_D ~14 nM and K_D ~21 nM, respectively). High-affinity binding between SsEno and fibronectin is consistent with other bacterial fibronectin-binding adhesins, including F1 and F2 of *S. pyogenes* (16, 31). These results support our hypothesis that the specific interaction between SsEno and fibronectin may be biologically significant. Both ELISA and SPR also demonstrated, as previously described for other pathogens, that the interaction between rSsEno and plasminogen is specific, suggesting that this interaction is also a biologically significant event. This provides further evidence to support the previous finding that bacterial enolases interact with plasminogen with high affinity (2, 28). In fact, the low nanomolar affinity constant between SsEno and plasminogen obtained in this study agrees with values obtained for adhesion of *S. pyogenes* (48) and *S. pneumoniae* (7) enolases for plasminogen.

Plasminogen-binding interactions were originally described as mediated by recognition of the C-terminal lysine residues of eukaryotic and prokaryotic enolases by the lysine binding sites of plasminogen (52). Later, Bergmann *et al.* (7) identified another internal plasminogen-binding site in the pneumococcal enolase (FYDKERKVY) that is located on the outer surface of the octameric molecule (15) and where the lysines and glutamic acid are important for plasminogen binding (6). Importantly, SsEno possesses both plasminogen-binding motif (M. Esgleas and M. Gottschalk unpublished observations) which could explain the similar nanomolar affinity constant of both *S. pneumoniae* (7) and *S. suis* enolase to plasminogen. Binding of plasminogen to *S. suis* enolase is inhibited by the lysine analog EACA, which indicates involvement of the lysine residues in this binding activity. Interestingly, SsEno adhesion to fibronectin is also decreased by EACA, indicating that lysines are also important for its fibronectin-binding activity. However, further research is required to determine the specific domain(s) and the individual amino acids that participate in the binding of SsEno to fibronectin.

As mentioned above, the pathogenesis of *S. suis* infection is poorly understood at present. Once in the bloodstream, *S. suis* resists phagocytosis and killing by neutrophils and monocytes (11, 12, 56, 58). In the event that *S. suis* fails to cause acute fatal septicaemia, bacteria are able to reach

the CNS via different mechanisms that are only partially elucidated, such as adhesion to, with or without toxicity, and invasion of brain microvascular endothelial cells (BMEC) (4, 13, 66) and/or choroid plexus epithelial cells (63, 64). In fact, interactions of *S. suis* with both fibronectin and plasminogen may play a role in some of these mechanisms. For example, fibronectin-binding proteins of streptococci and staphylococci have been reported to mediate bacterial adhesion to and invasion of host cells (46, 59, 65). In the case of *S. suis,* it has been recently demonstrated that *S. suis* adhesion to and intracellular invasion of PBMEC increases more than 500% and 700%, respectively, when bacteria are precoated with fibronectin (67). Although the mechanisms by which fibronectin-binding proteins trigger the internalization of bacteria by mammalian cells are not completely understood, it has been suggested that binding of fibronectin to a host integrin might initiate a complex cascade of cell signaling that leads to reorganization of cytoskeletal components and consequent internalization of the bacteria (54). Accordingly, *S. suis* invasion of endothelial cells is actin-dependent (66). In the present study, a role of SsEno in the adhesion to and invasion of these cells has been shown by blocking the protein using affinity purified anti-SsEno IgG. Since these studies have been carried out with cell monolayers, the fibronectin-binding activity of SsEno might explain this inhibition. It might be hypothesized that *S. suis* enolase is an important receptor for plasma fibronectin, which may increase bacterial adhesion to host cells. Another mechanism consisting in a certain adhesion of *S. suis* enolase to cellular fibronectin present in endothelial cells can not be completely ruled out. However, further studies have to be carried out to demonstrate such an interaction. Although no increase of adhesion/invasion to PBMEC were observed when increased concentration of plasminogen were added (M. Esgleas and M. Gottschalk unpublished observations), a certain role of the plasminogen-binding activity of SsEno (and GAPDH) in vivo, cannot be discarded. In fact, it has also been demonstrated that plasminogen adhered to *S. suis* is able to acquire plasmin activity when co-incubated with human BMEC (24). As a consequence of plasminogen activation on bacterial surfaces, bacteria become armed with the broad substrate spectrum proteolytic potential of plasmin that is not susceptible to regulation by host-derived inhibitors (35). The capture of plasminogen by adhesins such as SsEno and GAPDH and its convertion to plasmin has previously been described for other pathogens (2, 28) and can be used to facilitate bacterial penetration through biological membranes such as the BBB and therefore could represent an important determinant of virulence. The role of SsEno in studies using endothelial cells monolayers in a Trans-well system is currently under investigation in our laboratory.

In conclusion, we have demonstrated for the first time that *S. suis* α-enolase has not only a high affinity for plasminogen, as demonstrated in other bacterial enolases, but also a similarly high affinity for fibronectin. Moreover, we have shown that this protein is a new *S. suis* adhesin that participates in bacterial adhesion to and invasion of endothelial cells. Our results indicate that

SsEno of this human and porcine pathogen could be a key molecule in pathogenesis by facilitating bacterial interactions with host cells. This is in agreement with two recent studies that describes *S. suis* enolase as an antigenic protein and a putative virulence factor of *S. suis* serotype 2 (22, 73). Mutants with defective export of enolase to the surface of the bacteria may give some insights into the role of this adhesin in the pathogenesis of *S. suis* infection.

ACKNOWLEDGEMENTS

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ARTICLE III

Immunisation with SsEno fails to protect mice against challenge with *Streptococcus suis* serotype 2

Miriam Esgleas, Maria de la Cruz Dominguez-Punaro, Yuanyi Li, Josée Harel, J. Daniel Dubreuil and Marcelo Gottschalk

Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP) and Centre de Recherche en Infectiologie Porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, Québec, Canada.

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Role of the candidate in the conception of this article:

I am the main author of this article.

SUMMARY

In our ongoing efforts to develop a vaccine against *Streptococcus suis* infection, we tested the potential of *Streptococcus suis* enolase (SsEno), a recently described *S. suis* adhesin with fibronectin-binding activity, as a vaccine candidate in a mouse model of *S. suis*- induced septicemia and meningitis. Here, we show that SsEno is highly recognized by sera from convalescent pigs and is highly immunogenic in mice. Subcutaneous immunization of mice with SsEno elicited strong immunoglobulin G (IgG) antibody responses. All four IgG subclasses were induced, with IgG1, IgG2a and IgG2b representing the highest titers followed by IgG3. However, SsEno-vaccinated and non-vaccinated control groups showed similar mortality rates after challenge infection with the highly virulent *S. suis* strain 166'. Similar results were obtained upon passive immunization of mice with hyperimmunized rabbit IgG anti-SsEno. We also showed that anti-SsEno antibodies did not increase the ability of mouse phagocytes to kill *S. suis* in vitro. In conclusion, these data demonstrate that although recombinant SsEno formulated with Quil A triggers a strong antibody response, it does not confer effective protection against infection with *S. suis* serotype 2 in mice.

INTRODUCTION

Despite increasing research in recent years, *Streptococcus suis* continues to cause a variety of diseases in pigs worldwide, including septicemia, meningitis, arthritis and endocarditis (14). Among the 35 serotypes described, serotype 2 is considered the most virulent and is most frequently isolated from diseased pigs (14). In addition, *S. suis* has also been described as an important zoonotic agent, especially in Europe and Asia (22). Human infections with *S. suis* are most frequently manifested as purulent meningitis, but septic shock with multiple organ failure, endocarditis, pneumonia, arthritis, and peritonitis have also been reported (22).

The pathogenesis of *S. suis* infection is not fully understood. In swine, the potential portals of entry for *S. suis* are the palatine and pharyngeal tonsils and thereafter bacteria can spread via the hematogenous or lymphogenous route (23). Once in the bloodstream, *S. suis* has to resist phagocytosis and killing by phagocytic cells to cause acute septicemia that may lead to septic shock. Bacteria can reach different organs, including the central nervous system (CNS), via different mechanisms that are only partially elucidated to date (10).

Several potential virulence factors have been implicated in the infection process. The most promising virulence factors to date are the capsule polysaccharide (CPS) and serum opacity factor (OFS) (1), as isogenic mutants lacking either of these factors are rapidly cleared and eliminated from circulation (1, 6, 29). However, some non-virulent strains are also encapsulated or have allelic variations of OFS (30), indicating that virulence of *S. suis* likely involves multiple factors (10). Other virulence candidates have been proposed, but most of them are not present in all virulent strains or are present in non-virulent strains (11). So far, whole cell vaccines or bacterins (commercial and autogenous) have been used in the field to prevent *S. suis* disease, however, with disappointing results (13, 18). Vaccination with these bacterins does not induce high levels of antibodies and causes, at most, serotype-specific responses (14). We recently identified SsEno, a new *S. suis* surface fibronectin-binding protein (9) that might participate in the pathogenesis of *S. suis* infection by mediating bacterial attachment to and internalization into brain microvascular endothelial cells (BMEC) (9). SsEno might be an attractive vaccine candidate against *S. suis* infections as it possesses highly conserved epitopes (8) and is expressed at the surface of all *S. suis* serotypes described to date (9). In addition, a recent study identified *S. suis* enolase as an important antigenic protein that contributes to the virulence of *S. suis* (16). The objective of this study was to determine whether the immune response induced by immunization with purified SsEno can confer protection against challenge with the homologous strain of *S. suis* serotype 2 in a mouse model of infection.

METHODS

Bacterial strains

S. suis serotype 2 highly virulent strain 166' (2) was kindly provided by Dr. M. Kobisch, AFSSA, Ploufragan, France. Working log-phase cultures were prepared in Todd Hewitt broth (THB) (BD, Sparks, MD) as previously described (7). Growth was allowed until the suspension reached approximately 5 x 10^8 CFU ml^{-1}. Final inoculum corresponded to 10^7 CFU ml^{-1} (for pigs) and 10^8 CFU ml^{-1} (for mice). *Escherichia coli* strain BL21DE3 (Novagen, Madison, WI) was used for expression experiments as described elsewhere (9).

Cloning and expression of the α-enolase gene

Cloning and purification of SsEno were performed as previously described (9). Briefly, the coding sequence of SsEno (*S. suis* Enolase) was amplified by PCR using chromosomal DNA from *S. suis* SS166 as template and the complete gene was cloned into pET-32a vector (Novagen, Madison, WI). The plasmid pET-32a-SsEno was introduced into *E. coli* Bl21DE3 for an IPTG-inducible expression of recombinant *S. suis* enolase (rSsEno). The His-tagged fusion protein was purified by chromatography under native conditions on HisTrap according to the manufacturer's instructions (Amersham Biosciences AB, Uppsala, Sweden). Protein concentrations were determined by the Lowry method (21). Prior to vaccination assay, the purity (>95%) of recombinant SsEno preparations was determined by scanning densitometry of the protein on an SDS-PAGE gel stained with Coomassie blue and with a silver-stained SDS-PAGE gel (data not shown) as previously reported (9).

Presence of anti-SsEno antibodies in convalescent animals

All animal experiments were conducted according to the guidelines and protocols set forth by the Canadian Council on Animal Care and approved by the Université de Montréal committee on animal care. Seven 4-week-old piglets from a herd free of *S. suis* serotype 2 disease were infected intravenously with 10^7 CFU from a log-phase culture of *S. suis* strain 166'. Animals were monitored for clinical signs and treated with antibiotics if needed to avoid death. Serum samples were taken befor and three weeks post-infection. Antibody titers against rSsEno from these convalescent pig sera were measured by direct ELISA. Maxisorp® flat-bottom microtiter 96-well plates (Nunc, VWR, Mississauga, ON, Canada) were coated overnight at 4°C with 5 μg ml^{-1} of purified recombinant SsEno. The plates were further incubated with 1/1000 dilution of pig sera and bound antibodies were detected by incubation with peroxydase-conjugated goat anti-swine IgG (Jackson Immunoresearch Laboratories West Grove, PA) for 1 h at room temperature. The plates

were developed with TMB substrate (Zymed, S. San Francisco, CA, USA) and absorbance was measured at 450 nm.

Active protection assay of mice with rSsEno

Six-week-old female CD-1 mice (Charles River Laboratories, Wilmington, MA, USA) were immunized subcutaneously twice, one week apart, with either 20 µg of purified SsEno mixed with 20 µg of Quil-A adjuvant (Brenntag Biosector, Frederikssund, Denmark) or 20 µg of Quil-A only as a control in 100 µl of phosphate-buffered saline (PBS) per mouse. Ten days after the second vaccination, animals were challenged intraperitoneally with 10^8 CFU per mouse of log-phase *S. suis*, strain 166' in 1 ml of THB. Sera collected from each mouse before immunization, before the second dose and before challenge infection were assayed for anti-SsEno antibody titers by ELISA, as described below. Mice were monitored daily for clinical signs such as abnormal behavior (hyperexcitation, episthotonus, opisthotonus, bending of the head toward one side, walking in circles or strong locomotive problems), rough hair coat, ataxia and mortality until day 10 post-infection (7). This mouse model of infection was recently used to reproduce septic shock and meningitis that might be consider to be similar to those induced by *S. suis* in pigs (7, 14).

Passive protection of mice with rabbit antibodies against rSsEno

Groups of 12 female CD-1 mice (Charles River, 6 week old) were injected intraperitoneally with 0.5 ml of rabbit anti-rSsEno serum (9) or 0.5 ml of normal rabbit serum as a control. Hyperimmune sera against rSsEno was produced as previously described (20) and the titer (<1/100 000) evaluated by ELISA (28). Three hours later, 10 mice per group were injected intraperitoneally with 10^8 CFU per mouse of log-phase *S. suis*, strain 166' in 1 ml of THB. Sera were collected 24 h after the serum administration to measure anti-SsEno antibodies by ELISA assay as described below. Mice were monitored daily for weight loss, clinical signs and mortality.

Determination of active and passive antibody titers in mice by ELISA

Titers of SsEno-specific total IgG in mice sera were determined by ELISA as previously described (20). Briefly, Polysorb plates (Nunc-Immunoplates, Rochester, NY, USA) were coated overnight at 4°C with purified recombinant SsEno. Due to an extremely high antibody response (see below), and comparing to what was used to measure antibodies in swine, a significant reduced concentration of SsEno (0.3 µg ml^{-1}) was used to coat the plates. After incubation with serial dilutions of test, bound antibodies were detected by incubation with peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3 antisera (Serotec, Kidlington, Oxford, UK) for 1 h at room temperature. For determination of antibodies titers in the passive protection assay, the same

protocol was used but with peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) as a secondary antibody. Plates were then developed as described above. The antibody titer was considered as the serum dilution that resulted in an OD_{450} reading of 0.1 after subtracting background.

Killing of S. suis serotype 2 strain 166' by mouse phagocytes in the presence of anti-SsEno antibodies

In vitro killing of *S. suis* serotype 2 strain 166' by mouse phagocytes in the presence of polyclonal anti-SsEno antibodies was measured as described previously (17, 26) with some modifications. Briefly, 0.3 ml (from 5 different animals) of freshly heparinised blood was mixed with 0.5 ml of appropriately THB diluted bacteria (200-500 CFU ml^{-1}) in the presence of 25 $\mu g\ ml^{-1}$ of protein G purified rabbit anti-SsEno antibodies (9). Similar concentrations of purified normal rabbit antibodies were used as negative control and of purified hyperimmune rabbit anti-*S. suis* (whole cell) antibodies as a positive control, as previously described (4). Mixtures were incubated at 37°C for 3 h with constant slow rotation (17, 26). At the end of the incubation, an appropriately diluted aliquot was plated onto THB agar and incubated overnight at 37°C to count surviving bacteria. Results are expressed as mean percentage ± standard deviation of bacterial survival, with survival of bacteria opsonised with normal rabbit antibodies considered as 100%. Data are representative of four independent experiments. An internal control for the growth of *S. suis* serotype 2 strain 166' in the presence of the different conditions was also included. Briefly, the same concentration of diluted bacteria (200-500 CFU ml^{-1}) were grown in THB in the presence of 25 $\mu g\ ml^{-1}$ of the different purified antibodies or no antibodies for 3 h in stationary conditions and the number of CFU in each condition were counted.

Statistics

Antibody titers and percentage of killed bacteria of experimental groups were compared using student's *t*-test (*** $p<0.0005$,** $p<0.005$, * $p<0.05$). Survival curves were evaluated using the Kaplan-Meier method and the significance of the difference was tested using the Log-rank test.

RESULTS

Immunogenicity of SsEno in pigs

None of the seven animals used in this study had SsEno-specific antibodies before the experimental infection (Fig. 1), as only background values were detected in these animals. In contrast, pigs infected with a log-phase culture of *S. suis* strain 166' showed significant anti-SsEno-IgG responses at 3 weeks post-infection (Fig. 1).

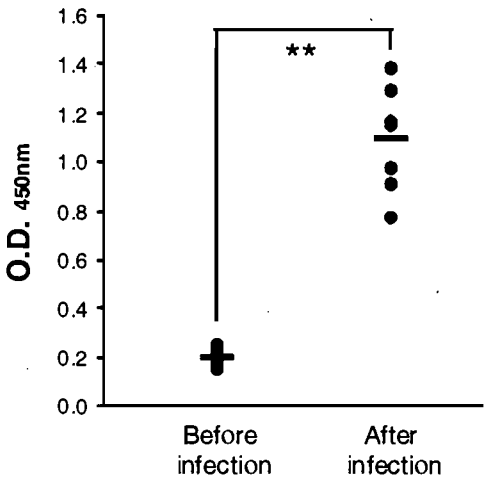

Fig. 1. ELISA detection of antibodies against SsEno in sera from pigs before and after challenge with *S. suis* serotype 2. Plates were coated with rSsEno and then incubated for 1.5 h with a dilution of 1/1000 of swine sera (before and after challenge). Mean values (line) comparing sera from non-infected ($n = 7$) and infected pigs ($n = 7$) are shown. **, $P<0.005$

SsEno-specific IgG and IgG subclasses in active protection assay

There were no SsEno-specific antibodies in sera from any of the animals before the first vaccination (Fig 2(a)). Immunization of mice with SsEno elicited a strong antigen-specific response. At 14 days after the first vaccination, SsEno elicited a significant IgG response that was further increased after the second immunization (Fig. 2(a)). Analysis of sera demonstrated that although SsEno-immunized animals produced all IgG subclasses, IgG1, IgG2a and IgG2b responses were predominant followed by IgG3 (Fig. 2(b)). In contrast, animals vaccinated only with adjuvant did not show any antibody responses (Fig. 2(a) and 2(b)).

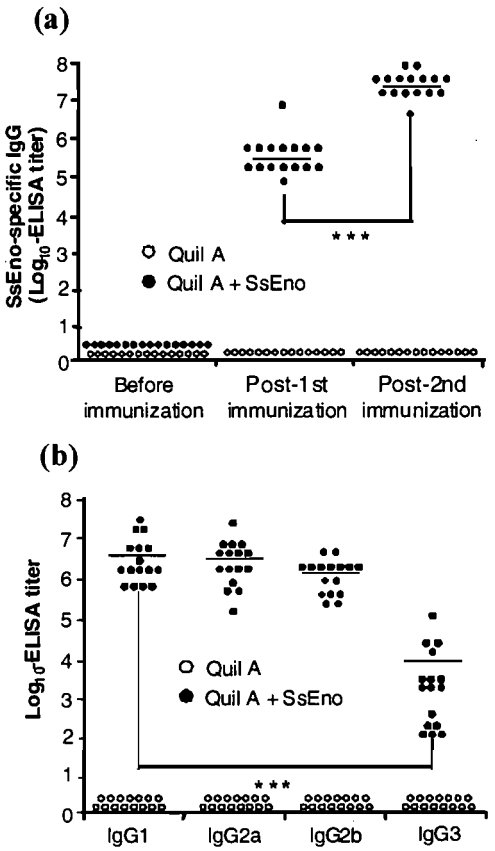

Fig. 2. Serum antibody responses in mice immunized with Quil A (open cycles) alone or Quil A plus recombinant SsEno (solid cycles). (a) Total SsEno-specific serum IgG. (b) IgG subclasses in sera at 14 days after the second immunization (before challenge). Antibody titers of individual mice are shown, with the average titer represented as a bar. (n=16 for SsEno-immunized animals; n=15 for control animals). ***, $P<0.0005$.

Clinical signs and mortality in the active protection assay

A few hours after challenge infection with *S. suis* serotype 2 strain 166', all mice (control and SsEno immunized group) exhibited clinical signs, such as ruffled hair coat suggesting fever and slow response to stimuli. From day 0 to 3 post-infection, 25 % of immunized animals died from septicemia (Fig. 3(a)). After day 3 post-infection, 58 % of the immunized animals that survived to septicemia developed severe CNS signs such as running in circles and opisthotonos and died or met criteria for euthanasia due to the severity of their condition (Fig. 3(a)). Similar results were observed in the control group; although all the animals resisted the septicemic phase, 33 % of animals died after day 3 post-infection due to meningitis (Fig. 3(a)). In all dead animals, *S. suis* serotype 2 was isolated from different organs (data not shown).

During the septicemic phase, the immunized group lost ~15 % of their body weight (Fig. 3(b)). Similar results were seen in the control group (Fig. 3(b)). In both groups, animals that survived the septicemic phase were able to recover their initial body weight at the end of the experiment (Fig. 3(b)).

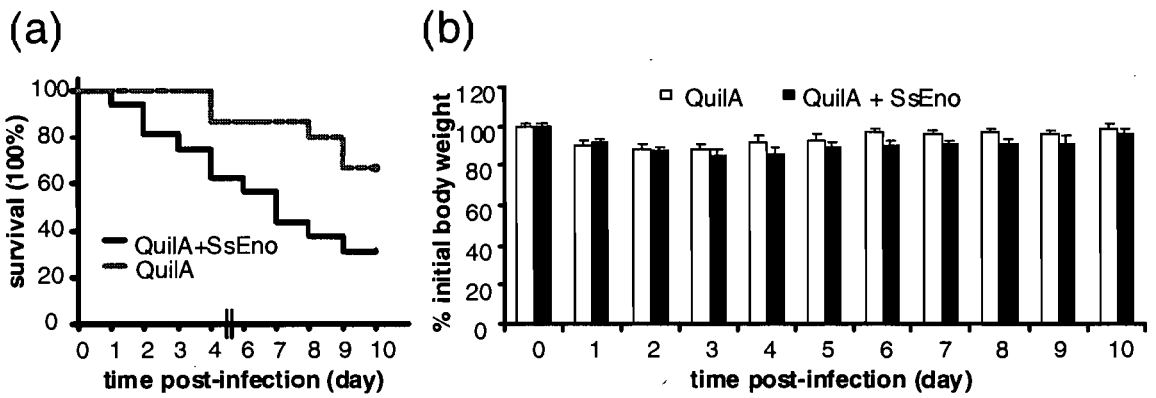

Fig. 3. Survival (a) and weight lost (b) of mice in the active protection assay. Mice were first immunized with Quil A (n=14, white) or Quil A-rSsEno (n=15, black) and then challenged with 10^8 CFU ml^{-1} of *S. suis* 166'. Survival rate (a) and % of initial body weight (b) were monitored for 10 days. Body weight of each mouse was measured every day.

Clinical signs and mortality in the passive protection assay

A few hours after infection, passively immunized animals presented similar clinical signs as those vaccinated in the active protection assay. During the first 12 h, 30% of animals immunized with anti-SsEno antibody died from septicemia compared with 70% of the control group. However, all passively immunized animals as well as control mice died within the first 6 days post-infection from either septicemia or meningitis (Fig. 4). Analysis of sera clearly indicated that there was a high titer of rabbit anti-SsEno only in the passive immunized group (data not shown).

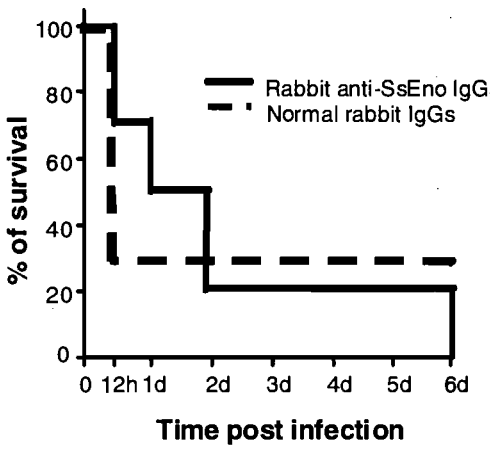

Fig. 4. Survival of mice in the passive protection assay. Mice were immunized with rabbit anti-SsEno antibodies (n=12) or control rabbit antibodies (n=12). Mice were then challenged intraperitoneally with 10^8 CFU ml^{-1} of *S. suis* serotype 2 strain 166' and survival was monitored for 10 days.

Killing of *S. suis* serotype 2 strain 166' by mouse phagocytes

To further evaluate the bactericidal/opsonic capacity of anti-rSsEno antibodies, a killing assay was performed using mouse whole blood. As shown in Fig. 5, *S. suis* treated with normal rabbit IgGs grew well in mouse blood (negative control). In contrast, *S. suis* treated with rabbit anti-whole *S. suis* IgGs was rapidly killed as expected (positive control). Incubation of *S. suis* with affinity purified anti-SsEno IgG antibodies did not enhance bacterial killing, confirming results obtained in the passive protection assay.

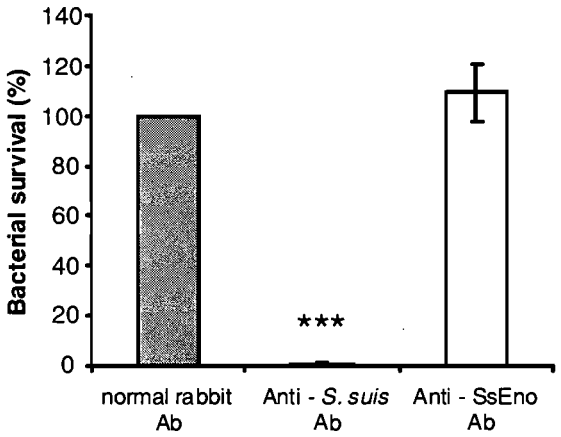

Fig. 5. Effect of antibodies on opsonophagocytic killing of *S. suis* strain 166' by mouse phagocytes. Antibodies were purified from sera of a rabbit immunized with SsEno. Antibodies from a normal rabbit and from a rabbit hyperimmunized with whole *S. suis* serotype 2 were used as negative and positive controls, respectively. Data are expressed as mean percentage ± standard deviation of bacterial survival, with survival of bacteria opsonised with normal rabbit antibodies considered as 100%. Data are representative of four independent experiments. ***, $P<0.0005$.

DISCUSSION

Several approaches have been used to develop vaccines for *S. suis*. However, little success has been achieved thus far because the protection elicited was either serotype or strain dependent, and results in most instances have been equivocal (12). For example, killed whole cells or live avirulent vaccines can provide partial protection but only with repeated immunization (3, 33). Subunits protein-based vaccines have also been tested. using virulence markers such the hemolysin (15), the muramidase-released protein and extracellular protein factor (33) that have been shown to protect pigs from homologous and heterologous serotype 2 strains. However, their use is hindered by the fact that a substantial number of virulent strains in some geographical regions do not express these proteins (10). More recently, a surface expressed protein (SAO) has been observed to confer protection against experimental infection in mice and pigs (19). However, as this protection is not complete, other proteins could be combined with SAO to optimize protection.

Fibronectin-binding proteins have been suggested as potential vaccine targets for preventing bacterial infections because antibodies directed against such adhesins may prevent bacterial attachment and also enhance opsonization and killing by leukocytes (27, 34). In this study, we tested the potential of the recently described fibronectin-binding protein, SsEno, as a vaccine candidate in a mouse model of *S. suis*-induced septicemia and meningitis (7). SsEno is a good vaccine candidate based on the following characteristics: i) it is present at the surface of all 35 different *S. suis* serotypes (9); ii) it contributes to *S. suis* adhesion to and invasion of host cells (9); and iii) it is a highly conserved protein (8). In addition, we further showed that sera from convalescent animals strongly recognize this protein, suggesting expression of SsEno in vivo. Very recently, another study also suggested an important role of enolase in the virulence of *S. suis* (16). However, the potential of this protein to confer protection against other pathogens is controversial. Mice immunized with antibodies against *Plasmodium falciparum* enolase are protected from challenge infection with a lethal mouse malaria strain (25). In contrast, recombinant enolase from *Candida albicans* induces only modest protection against disseminated candidiasis (24). To our knowledge, a protection test against streptococci using enolase as an antigen to elicit an immune response has not been reported yet.

To evaluate SsEno as a vaccine candidate against *S. suis* infection, we used a highly virulent strain for swine in a well standardized mouse model, which presents two different phases: a) a septicemic phase, (24-48h post-infection) and, b) a meningitis/encephalitis phase, where animals that survive septicemia will subsequently die from a serious infection of the CNS (7). Our results indicated that the antibodies actively elicited by SsEno at the concentration used in

combination with Quil-A adjuvant was not protective from either phase of challenge infection with *S. suis*. The Th1-type responses and IgG subclasses induced by the SAO protein are two main components of host immunity against *S. suis* infection (19). In this study, all SsEno-specific IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) were induced, with IgG1 and the Th1-type antibodies, IgG2a and IgG2b, as the predominant subclasses. Therefore, other components of the host immune response besides non-opsonic antibodies may be necessary for effective protection against *S. suis* infection. We attributed the fact that the vaccinated group presented a higher level of mortality than the control group to a simple variation in an animal assay. Although it would have been tempting to interpret the results as a possible role of antibodies against enolase playing a certain role in autoimmunity as described (31), animals from both groups were perfectly healthy at the moment of challenge.

To confirm these results, we tested whether rabbit anti-SsEno serum provides passive protection against challenge infection with the same strain of *S. suis* in mice. As expected, and although a slight delay in the appearance of severe clinical signs, passive immunization with anti-SsEno serum did not confer significant protection against *S. suis* infection. As it has been shown that antibodies derived from hyperimmune sera against whole bacteria (including antibodies against the capsule) can protect mice against infection (5) and induce bacteria killing (4), it is possible that anti-SsEno antibodies are not opsonic, allowing bacteria to reproduce in high numbers to cause disease. On the other hand, antibodies against other fibronectin-binding proteins have already been described to enhance phagocyte killing in other bacterial species (27). To test this, we evaluated the capacity of the purified anti-SsEno IgGs to opsonize bacteria and promote their killing by murine phagocytes. Although most of the produced antibodies were of the Th1 type, results obtained in this study showed that the anti-SsEno IgG subclasses were not able to induce *S. suis* killing by mouse phagocytes.

Since anti-SsEno has been shown to reduce bacterial adhesion and invasion to BMEC in vitro (9), we had expected a certain degree of protection against the CNS phase of infection. However, SsEno is not the only receptor involved in such process (32). It is possible that the high concentration of *S. suis* in blood, as a consequence of a lack of bacterial killing by leukocytes, may overcome the partial inhibition of bacterial-BMEC interactions mediated by anti-SsEno antibodies.

In summary, although we demonstrated that SsEno elicits an important antibody response in convalescent pigs and immunized mice, this response as evaluated in the present study is inadequate for effective protection against *S. suis* infection.

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IV. DISCUSSION

Streptococcus suis is a well-recognized worldwide swine pathogen of emerging clinical significance in most countries with intensive swine industry (37). *S. suis* infections in pigs are associated with different clinical diseases such as meningitis, arthritis, endocarditis, septicemia and occasionally, other infections (37). Clinically healthy pigs can carry *S. suis* in their nasal cavities, tonsils, and upper respiratory tracts, contributing to the dissemination and transmission of this pathogen (29). In addition to its significant sanitary and economic impact in the swine industry, *S. suis* is also a zoonotic agent, responsible for septicemia and meningitis in humans (55). *S. suis* normally affect people in close contact with diseased pigs or with their pork-derived products (55). The number of *S. suis* infections in humans and the number of death caused by this pathogen dramatically increased in the last years and, although the hard work realized to eliminate this bacterial infection, the control of the disease is hampered by the lack of effective vaccines (55). In addition, as most of the pathogenic and etiologic agents of deadly diseases, *S. suis* is acquiring resistance to currently used drugs indicating that the development and formulation of vaccines is extremely necessary (36).

Discovering virulence factors of pathogenic bacteria is an important key in understanding pathogenesis and for identification of targets for novel drugs and design of new vaccines (101). Bacterial virulence factors can be divided into several groups on the basis of the mechanism of virulence and function (101). These are (i) membrane proteins, which play roles in adhesion, colonization, and invasions, promote adherence to host cell surfaces, are responsible for resistance to antibiotics, and promote intercellular communication, (ii) polysaccharide capsules that surround the bacterial cell and have anti-phagocytic properties, (iii) secretory proteins, such as toxin, which can modify the host cell environment and are responsible for some host cell–bacteria interactions, (iv) cell wall and outer membrane components, such as lipopolysaccharide and lipoteichoic acids, and finally (v) other virulence factors, such as biofilm forming proteins. Biofilm formation confers a selective advantage for persistence under environmental conditions and for resistance to antimicrobial agents and also facilitates colonization in the host by the bacteria.

As described in this work, several putative virulence factors have been identified for *S. suis* (29). The capsular polysaccharide and the OFS are so far the only proven critical virulence factors since isogenic knock-out mutants for these proteins were shown to be avirulent and cleared from circulation rapidly in different models of infection (3, 10, 83). In addition, a muramidase-released cell-wall protein and an extracellular protein factor were originally associated with virulent serotype 2 strains. However, isogenic mutants lacking these two proteins appeared to be as virulent as the wild-type strain. It is therefore not clear if these proteins are virulence factors per se or that their synthesis is only coincidentally associated with virulence (84). *S. suis* also produces a hemolysin,

member of the thiol-activated cytolysin family of toxins, named suilysin (40). The roles of these toxins in pathogenesis are varied and not fully understood, however, they all lyse eukaryotic cells that contain cholesterol (40). It has been described that suilysin is also able to lyse different type of host cells (29). Possibly, it might be involved in the dissemination of bacteria through the organism by disrupting several host barriers by affecting the cells forming these barriers (93). In addition, several other *S. suis* proteins that have a role as adhesins have also been described as a potential virulence factors (29). Finally, it has been recently described that, as for other bacterial pathogens such as *S. aureus* (64), *S. suis* is able to produce a biofilm which protect biofilm-grown *S. suis* from some antimicrobial agents such as penicillin G (6). It is important to note that most of these virulence factors have been tested as vaccine candidates against *S. suis* infections, however, the results obtained up to day have not been very succesful.

All these described (potential) virulence factors have a role in the pathogenesis of the infection caused by *S. suis*. However, as described in this work, the pathogenesis of *S. suis* infection is a complex process where many steps, most of them still unknown, are probably involved (29). It has been demonstrated that *S. suis* is a natural host of the tonsils of pigs and that some of the bacteria are able to cross the epithelial respiratory barrier and reach the bloodstream (29). In humans, bateria reach directly the bloodstream by lesions or minor abrasions in the skin (37). Once in the circulation, *S. suis* is able to evade the immune system permitting its dissemination through the organism (8, 78, 79). In some cases, bacteria provoke septicemia and death (85). However, in case the host survives septicemia, bacteria are able to arrive to the target organs. In fact, *S. suis* is frequently isolated from brains of diseased pigs with clinical meningitis indicating that this pathogen has passed from the bloodstream to the CNS crossing the BBB (22).

Many questions about the different steps of the pathogenesis of the infection caused by this pathogen and the molecules involved in those steps are still unresolved (29) (Fig 1). The first unresolved question is which mechanisms/molecules permit *S. suis* to evade the immune system of the host and travel in the bloodstream to finally arrive to the BBB, the other important question is how bacteria present at low levels on mucosal surfaces are able to traverse the host barriers such as the respiratory barrier or the BBB to develop disease. (29). Many studies have been done in the last years to try to answer these question marks. On one hand, several studies reported that, in contrast to nonencapsulated mutants, encapsulated *S. suis* is able to resist phagocytosis, indicating that capsule is an important factor that protect bacteria from phagocytosis (8, 78, 79). However, resistance to clearance from the bloodstream does not rely only on the presence of the capsule, since a well encapsulated avirulent strain is eliminated from circulation within 48 h, whereas a virulent strain can stay in relatively high numbers in the blood for more than 5 days (29). These results

indicate that other important molecules are also responsible for this immune system evasion and that further research is necessary to determine the mechanisms by which *S. suis* avoids phagocytosis. On the other hand, one of the most accepted proposed mechanisms used by this pathogen to cross the host barriers is the adhesion to and subsequent invasion of the host cells forming these barriers (88, 93). It is commonly believed that the adhesion of microorganisms to host tissues represents a critical first step in the process of microbial infections. In fact, non-adherent bacteria are readily eliminated from the body by such cleansing mechanisms as peristalsis and excretion (43). In contrast, bacteria that adhere to host tissues may remain extracellular or may be internalized into an intracellular compartment (43). This cellular invasion enables the bacteria to escape host defense mechanisms, persist in the host and finally to cross host barriers (43).

This ability to cross host barriers has been observed in several pathogens including Gram-positive and Gram-negative bacteria. Pathogens may cross the BBB transcellularly, paracellularly and/or by means of infected phagocytes (so-called Trojan horse mechanism) (47). Transcellular traversal of the BBB has been demonstrated for several bacterial pathogens, such as *E. coli*, group B Streptococcus, *S. pneumoniae*, *L. monocytogenes*, *Neisseria meningitidis*, *Mycobacterium tuberculosis* and fungal pathogens such as *C. albicans* and *Cryptococcus neoformans* (9, 46, 48). Paracellular penetration of the BBB has been suggested for the protozoans *Trypanosoma sp* (57). This pathogen normally uses the fibrinolytic system in which bacterial-adhered plasminogen is converted to plasmin that is able to degrade the tight junctions forming the BBB. This destruction of the cell junctions permits the pathogen to cross the BBB between cells. Finally, the last proposed mechanism is the Trojan horse mechanism or the modified Trojan horse mechanism where infected phagocytes carry the pathogen through the BBB (47). This mechanism has been suggested for different pathogens such as *L. monocytogenes* and *M. tuberculosis* (23). Other minor routes of bacterial entry into the CNS include mechanical spread from a contiguous source of infection such as sinusitis and mastoiditis. For example, *S. pneumoniae* has been shown to enter the CNS through non-haematogenous route in experimental animals after intranasal infection and otitis media (56, 92).

As it has always been observed in *S. suis* infected pigs with meningitis, it has been recently demonstrated that *S. suis* is found in the brain of mice with clinical signs of meningitis, indicating and corroborating that this bacteria is able to cross the BBB (22). Different mechanism used for *S. suis* to cross the BBB have been postulated. The first one is the adhesion to and invasion of BBB because although *S. suis* is a pathogen known to reside in extracellular niche, it has been demonstrated that *S. suis* has the ability to adhere to and, in some cases, invade different host cells including those forming the BBB (88, 93). In addition, it has also been postulated that *S. suis* could

also use the "modified Trojan horse mechanism" in which bacteria are able to cross the BBB adhered to phagocytes such as monocytes (78, 79). In both ways, the adhesion of *S. suis* to host cells is extremely important. However, although the routes of entry seem to be known, the bacterial molecules responsibles for these adhesions are in most of the cases unknown.

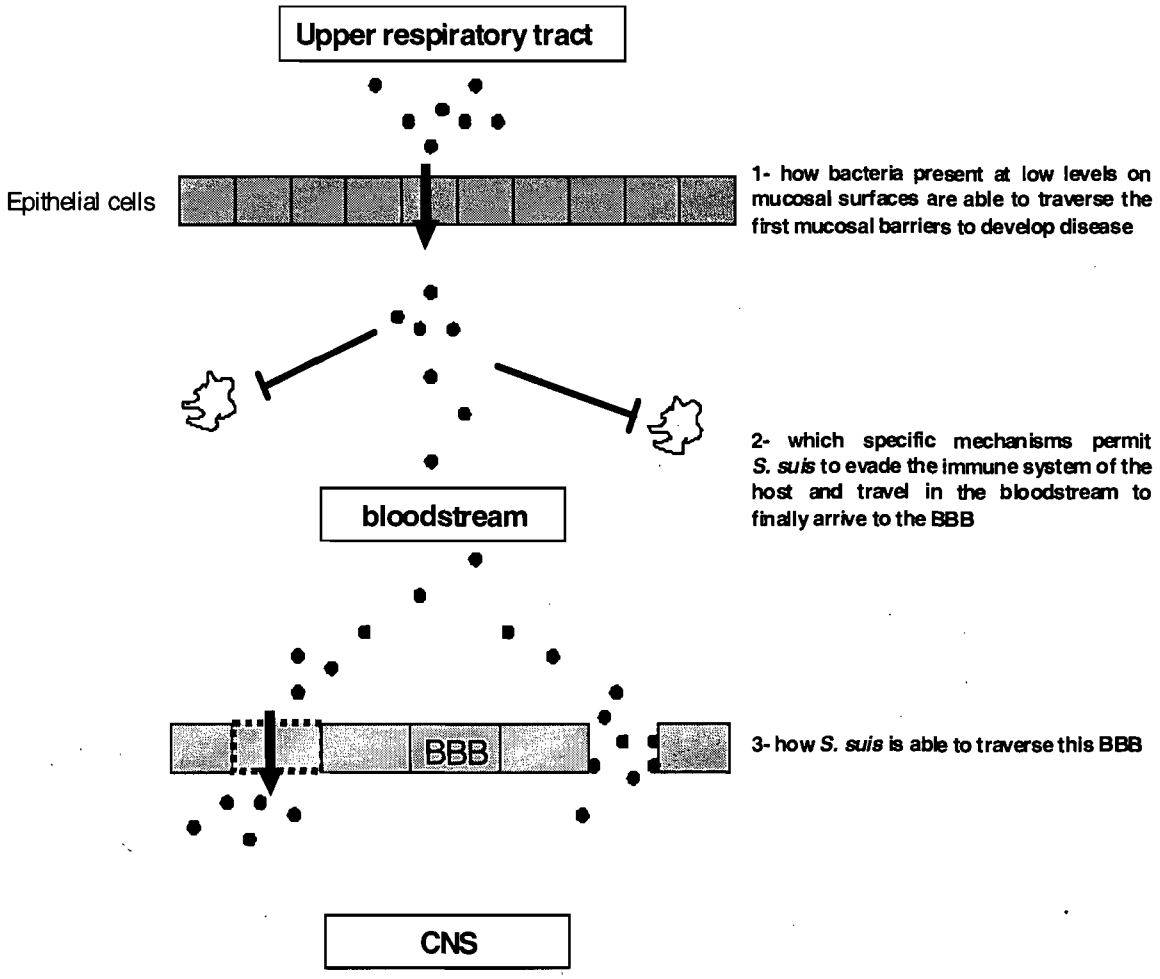

Fig 1. Important unresolved questions about the pathogenesis of the infection caused by *S. suis*. Fig modified from (29)

The adhesion of bacteria to the host cells mainly involves two molecules: the bacterial surface components, called adhesins, and the host receptors (43). One of the most known and studied host receptors is the extracellular matrix (ECM) (43). Host ECM components that are known to support bacterial adhesion include fibronectin, collagen, fibrinogen/fibrin, elastin, vitronectin, laminin (43). On the other hand, the subfamily of bacterial adhesins that bind to ECM molecules are collectively known as Microbial Surface Components Recognizing Adhesive Matrix

Molecules (MSCRAMMs) because more of the 90% of the adhesins responsibles for these adhesions are from this family of proteins (43, 70). However, and as it will be explained latter in this discussion, other bacterial adhesins subfamilies that participate in the adhesion of bacteria to ECM proteins have been reported in the last years (14).

Although a high number of microbes have been shown to bind different host ECM components (43, 62, 98), the adhesins responsible for these interactions have, in most cases, not been identified or characterized. In the case of *S. suis*, little was known, before this work, about the ability of this pathogen to bind to ECM proteins and the adhesins responsible for these adhesions. Only three works have been published about the ability of *S. suis* to bind to some ECM proteins. De Greef *et al.* identified in 1991 by "in vivo selected genes" the only fibronectin-fibrinogen-binding protein (FBPS) described for this pathogen before this work (19, 20). They demonstrated that the recombinant FBPS was able to adhere to human fibronectin and fibrinogen, two of the main components of the ECM, and that this protein seems to participate in the colonization of the organs specific for a *S. suis* infection in piglets, but not in the colonization of *S. suis* on the tonsils of piglets (19). Moreover, they showed that the *fbps* gene is present in all known serotypes of *S. suis* [except for serotypes 32 and 34 that up to now can be considered as *S. orisratti* (38)] (19). However, the expression of FBPS in all serotypes was not studied. Therefore, it is possible that although all strains, except for serotypes 32 and 34, possess the fbps gene, not all strains express FBPS. The second work about the adhesion of *S. suis* to some ECM protein describes a 52 kDa IgG-binding protein that reacts with a large variety of IgGs and with human IgA, but also with other human plasma proteins including the ECM protein fibronectin (80). However, the role of this protein in the pathogenesis of the infection caused by *S. suis* has never been studied so far. Finally, a last work about the adhesion of *S. suis* to fibrinogen has been recenty published. This work shows that fibrinogen enhances *S. suis* biofilm formation suggesting that *S. suis* has the potential to adhere to fibrinogen (6). Although these three works demonstrate that *S. suis* is able to bind to some ECM proteins, an extensive research about the capacity of *S. suis* to bind to the ECM and particularly to the different proteins conforming this ECM was need to better understand some of the steps of the pathogenesis of the infection caused by this pathogens and the molecules that are involved. For this reason the first objective of this work was to study the capacity of *S. suis* to adhere to some of the most important ECM proteins presents in the host.

Our results show, for the first time, that whole *S. suis* is able to specifically bind, in a dose, temperature and time-dependent manner, to different types of collagen and fibronectin, the 2 main components of the ECM. However, in this work, *S. suis* was not able to bind to any of the other ECM protein tested. It is important to note that, although *S. suis* is mainly a swine pathogen, the

protein used for this first objective were from human origin because they were the only commercially available. In this work *S. suis* was not able to bind to collagen type IV, elastin, vitronectin, fibrin, laminin and fibrinogen. Although, as explained above, De greef *et al.* described a *S. suis* fibronectin-fibrinogen-binding protein (19) and a recent work also demonstrated that *S. suis* enhances the formation of a biofilm using fibrinogen (6), we can not detect any adhesion of *S. suis* to fibrinogen. The possible cause for this lack of adhesion in our work could probably be the technique used in this study. The other two works described here used soluble fibrinogen whereas we bound fibrinogen to the ELISA plate well. The adhesion of fibrinogen to a surface can probably modify its conformational structure and, as a consequence, the fibrinogen adhesion sites for bacteria can be hidder. In fact, differences in adhesion depending on the conformation of fibrinogen have also been reported for other bacteria. For example, *S. gordonii* is able to bind to immobilized fibrinogen, but not to soluble fibrinogen (51). In conclusion, it appears likely that fibrinogen expresses determinants that are recognized by bacteria only depending in the conformational form of the protein.

As described before, we have demonstrated that *S. suis* is able to bind to collagen. Collagens are structural proteins of extracellular matrix that typically have triple helical domains of variable length (35). Collagens form, for example, the large fibers of connective tissues and networks in basement membranes, while some collagens are transmembrane proteins (35). The collagen family has been divided into two subgroups (35): i) the fibril-forming collagens with a long, continuous triple helix that gives the molecule a rigid, rod-like structure. These collagens form large fibrils that give structural integrity and tensil-strength to the tissues. Type I, II and V collagens are the most abundant protein in mammals forming extracellular matrix in bone, tendons, dermis etc, and ii) the network-forming collagens that have interruptions in their triple helix. Type IV collagen, belonging to this subfamily, is an important structural component of basement membranes.

Different studies have shown the presence of collagen-binding receptors on some species of bacteria. The Dr fimbriae found on uropathogenic *E. coli* strains with the serotype 075 have been found to bind type IV collagen (99), while the enteropathogenic *Yersinia enterocolitica* and *Y. pseudotubercolosis* adhere to various collagen molecules through the YadA protein (25). Within the genus *Streptococcus*, there are other species, mainly among the groups A, B, C, and G, which bind collagens (71). This binding has been postulated as a factor contributing to the development of a number of infections. For example, binding of *S. mutans* strains to collagen has been proposed to play a role in the pathogenesis of root caries (33), and the ability of *S. pyogenes* to bind collagen type IV may be an important virulence factor in determining post streptococcal glomerulonephritis

(49). In this work we demonstrated that *S. suis* binds to the most abundant collagen types in mammals, the types I, III, and V. However, and in contrast with other streptococci (49), *S. suis* has not the ability to bind to the collagen type IV. Differently to the other collagen types, collagen type IV is only found in the basement membrane of the cell (30) and, as a consequence of its localisation, it has a different structure (30). This structural difference might hide receptors required for *S. suis* binding and could be responsible for this dissimilarity in the adhesion. Interestingly, although *S. suis* is not able to bind directly to this collagen type IV, we have demonstrated that it has the potential to adhere to this protein via a surface-bound fibronectin mechanism. This fibronectin-mediated collagen recruitment has been previously described in other pathogens as a novel mechanism of colonization and immune evasion (21). In *S. suis* this mechanism could be of high importance since its route of entry in humans is through lesions or minor abrasions in the skin where the first proteins exposed to bacteria are the proteins forming thrombus, such as fibronectin, and the proteins of the basal lamina, including the type IV collagen (103). In addition, interaction via fibronectin with collagen found in specific tissues offers the pathogen multiple potential attachment sites such as fibronectin-mediated binding to collagen fibres that can allow the pathogen to colonize acellular tissue and collagen-binding integrins that may serve as subsequent receptors, thus rendering distinct cell types susceptible to *S. suis* attachment.

On the other hand, we have also demonstrated that *S. suis* also binds to plasma (soluble) and cellular (solid-phase attached) fibronectin. Other pathogenic microorganisms have been reported to interact differentially with soluble versus solid-phase fibronectin. *S. sanguis*, group B streptococci and Yersinia sp. bind fibronectin adherent to a solid phase but do not bind soluble fibronectin (77, 87). In contrast, *S. aureus* and *S. mutans* binds fibronectin in both phases (15, 73), which is similar to the situation with *S. suis*. It was speculated that the surface domains presented by soluble and solid-phase fibronectin differ considerably (90). Since soluble fibronectin is ubiquitous in body fluids, it is logical that bacteria might evolve fibronectin-binding proteins which are not saturated by fibronectin in plasma, so that they may attach directly to tissue- or foreign body-associated fibronectin (15). In addition, binding of soluble fibronectin may facilitate the extravasation or migration from oral compartments to distant regions of the body, and coating of the microorganism with soluble fibronectin may facilitate escape from host immune surveillance (15).

Similar to collagen, fibronectin is also a high-molecular-weight glycoprotein that binds to membrane spanning receptor integrins (91). This adhesion helps maintain the shape of cells by lining up and organizing intracellular cytoskeleton, but in addition, permit bacteria to invade the cells by activating the cytoskeleton of the cell via fibronectin-attached integrins (1). In addition to integrins, fibronectin also bind other extracellular matrix components such as collagen and fibrin

indicating that microorganisms may bind to circulating fibronectin and become attached to tissues through fibronectin-fibrin, fibronectin-collagen, or fibronectin-fibronectin interactions (96). In the case of *S. suis*, its binding to fibronectin could have different important functions in the development of the infection. First of all, it can impede that bacteria are rapidly cleared from the circulation since it permits bacteria to adhere to ECM-forming cells, secondary, it can hide important antigens impeding bacterial recognition by the immune system (antibodies and complement) from the host, and as consequence, its elimination (98). Finally, this adhesion can also permit bacteria to bind to cell receptors such as integrins, activating the host signal transduction for intracellular polymerization and reorganization of actin filaments (98) and, as consequence, can permit *S. suis* to cross host barriers such as BBB by invading the host cells forming these barriers. In fact, it has been demonstrated that invasion of *S. suis* to PBMEC is an actin-dependent process (94) indicating the participation of integrins, of some ECM proteins such as fibronectin and, of probably, a fibronectin-binding adhesin of S. suis in this process. It is important to remark that, although a fibronectin-fibrinogen-binding protein (FBPS) with capacity to bind to these ECM proteins has been described for *S. suis* before this work and a mutant lacking this protein was constructed (19), the ability of this mutant to bind to fibronectin compared to its parental strain has never been studied before. Our results indicate that the fbps knock-out mutant binds to fibronectin in a similar way than its parental strain. In addition, it has also been demonstrated that the isogenic mutant for this protein binds to PBMEC cells at similar levels as those presented by the wild-type parental strain (94). These results indicate that other fibronectin-binding proteins are implicated in the fibronectin-adhesion process of *S. suis*. This is not the first case that more than one fibronectin-binding protein is described for the same bacteria, for example, in the case of *S. pyogenes*, SfbI protein or protein F1 (34, 86), GAPDH (68), M3 (76), FBP54 (16), protein H (27), SfbII/OF (50), F2 (41), M1 (18) and PFBP (75) have been identified among others as *S. pyogenes* surface molecules that bind to fibronectin.

The bacterial ligands that allow binding to ECM proteins could be either a protein or other bacterial surface component (17). To determine which sort of molecules are responsible of the adhesion of *S. suis* to fibronectin and/or collagen, different treatments were done. The dramatic reduction in the binding of both, fibronectin and collagen, by trypsin-, pronase-, and proteinase K-treated bacteria indicates that, in the case of *S. suis*, these adhesions are mediated by a protein. This conclusion is supported by the fact that heat treatments (60°C and 100°C) also decreased binding to ECM proteins. In other species, it has been proposed that bacterial capsule could also participate in the binding between pathogens and cells, probably via its ECM proteins (97). However, our hypothesis that proteins, instead of capsule, are the principal responsible of *S. suis* adhesion to fibronectin and collagens is supported by the fact that an unencapsulated mutant binds more to these

proteins than its encapsulated parental strain. Moreover, this unencapsulated mutant also has the ability to bind to other ECM proteins such as vitronectin and laminin indicating that capsule could be hiding the adhesins responsible for these adhesions. These results confirm that capsule does not participate in the *S. suis*-ECM protein adhesion and that, as showed before, proteins are the major responsible for these adhesions. In addition, cross-inhibition was observed between soluble fibronectin and collagen indicating that fibronectin and collagen binding domains exist on the same or nearby bacterial surface adhesins. Similar results were obtained with GBS by Tamura and Rubens (87).

The second objective of this work was to identify and characterise the adhesins involved in the fibronectin and/or collagen binding activity of *S. suis*. Some bacterial adhesins are highly specific whereas other recognize more than one protein (98). In an attempt to identify some *S. suis* collagen-binding proteins we have identified by "plasmid rescue" (82) (Annex 6, Fig 2) eight different genes that seem to be implicated in this adhesion (Annex 6 Table 1). To date, the best studied collagen-binding protein of Gram-positive cocci is the Cna surface protein of *S. aureus* (74). The mature Cna protein (135 kDa) has a typical MSCRAMM structure (see below) containing two domains, the collagen type I-binding 500 aa A-region and a repeated B-region (74). However, in contrast to the fibronectin MSCRAMMs, no defined function has been described for the repeated domain of this collagen adhesin (74). In this protein, a 19-kDa subdomain within the A domain was found to be responsible for the majority of collagen binding activity (74). Although the eight genes identified in *S. suis* seem to participate in its collagen-binding activity, any of them has the typical bacterial collagen-binding protein structure described here neither any of them seem to participate in the export of the collagen-binding proteins to the surface of bacteria. These results indicate that other methods have to be used to identify the proteins responsible for the adhesion of *S. suis* to collagen and that, further research is necessary to determine the role of the genes identified in this work in the capacity of *S. suis* to bind to collagen.

On the other hand, the identification of a new *S. suis* fibronectin-binding protein was done by another approach. Briefly, a lysate of bacterial proteins was passed through a human fibronectin-coupled column and the proteins with affinity for fibronectin were eluted after washing the other *S. suis* molecules with no affinity for fibronectin from the column. The recovered *S. suis* fibronectin-binding proteins were then visualised with coomasie blue staining and the amino-terminal sequence of a predominant *S. suis* fibronectin-binding protein of about 52 kDa was sequenced. Although we expected for a typical bacterial fibronectin-binding protein with high homology with other described streptococci fibronectin-binding protein, the BLAST analysis of the amino-terminal sequence of this 52 KDa protein revealed that this protein is an α-enolase with more than 93% of

similarity with other described bacterial enolases (66). α-enolase is one of the key glycolytic enzymes (1) found generally in the cytoplasm, nevertheless, its presence on the surface of cells is not without precedent. An α-enolase has been identified on the surface of many eukaryotic cells such as on the surface of neuronal, cancer and some haemopoietic cells (54, 59, 63). In addition it has also been described in the surface of several types of bacteria, including streptococci and staphylococci, fungi and nematode (5, 44, 45, 60, 67, 81). The identity of this enzyme as α-enolase was confirmed by biochemical and a functionality tests. In fact we have demonstrated that this bacterial protein is able to convert the phosphoglycerate to phosphoenolpyruvate as it normally occurs in the cell glycolysis process (1). This new *S. suis* fibronectin-binding protein was named SsEno for *Streptococcus suis* Enolase. It is important to note that this is the first time that the fibronectin-binding activity of an α-enolase is described.

As SsEno is a predominant fibronectin-binding protein of *S. suis* and as *S. suis* is mainly a swine pathogen we decided to test the capacity of this new described protein to adhere to porcine fibronectin. However, as explained before, porcine fibronectin is not commercially available so we decided to create a new protocol to purify fibronectin from porcine blood plasma (Annex 1). Using the known high affinity of fibronectin for gelatine and a posterior HLPC purification (Annex1, Fig 1, 2 and 3) we obtained a high pure porcine blood plasma fibronectin (Annex 1, Fig 3). This protein was used to demonstrate that SsEno has not only high affinity for human fibronectin but also a similar high affinity for porcine fibronectin (Annex 1, Fig 4). These results indicate that this pathogen can use this ECM protein to enhance its infection in both, swine and human hosts. Interestingly, recent studies demonstrate that other described streptococcal enolases have not this ability to bind fibronectin (2), suggesting that interaction of enolase with fibronectin may have different function in *S. suis* than in other pathogenic bacteria.

The main function of surface enolases described before this work is their capacity to bind plasminogen (66). This feature was first demonstrated for eukaryotic surface enolases and later for prokaryotic enolases (66). We have demonstrated that, in addition to its fibronectin-binding activity, SsEno also binds to plasminogen as it has been previously described for all the other bacterial enolases (2). Interestingly, SsEno binds to fibronectin with a similar affinity than to plasminogen, indicating that both functions could be of high importance for this pathogen. However, in contrast with the *S. aureus* enolase (7), SsEno has not the ability to bind to laminin, corroboring that this protein can have different roles depending on the pathogen. As we observed cross-inhibition with soluble fibronectin and collagen in the ELISA assay of the first objective, we test the capacity of SsEno to adhere to collagen. However our results showed that this protein does not possess this

binding activity (Annex 5, Fig. 1) indicating that SsEno is not the surface protein responsible for the cross-inhibition between fibronectin and collagen.

We have also demonstrated that in addition to its fibronectin and plasminogen-binding activity, SsEno has also IgG-binding activity (Annex 4, Fig 1). This is not the first time that it is described that a fibronectin-binding protein has IgG-binding activity. In fact, the fibronectin-binding protein I (SfbI) of *S. pyogenes*, a main adhesin and invasion of this pathogen, is also able to bind to human, mouse, rabbit, pig and horse IgG (58). We have demonstrated in this work that SsEno is able to bind to porcine and human IgG and to IgY. This is the first time, to our knowledge, that the IgY-binding activity of a bacterial protein is described. It remains to be determined whether these IgG-binding activities of SsEno can contribute to the pathogenesis of the infection. It is commonly believed that the IgG-binding activities can favor bacterial immune evasion following infection by interfering with host clearance mechanisms (58). This hypothesis is supported by the fact that α-enolase is not only present in the cytosol and the surface of bacteria but it is also secreted by *S. suis*. This secrection can permit bacteria to evade host immunoglobulins since secreted SsEno binds to them before they recognize the pathogen. In addition, it has been demonstrated that *S. sobrinus* enolase stimulates an early production of interleukin-10, an anti-inflammatory cytokine, and not the pro-inflammatory cytokine IFN-γ indicating that, in this specie, enolase acts in the suppression of the specific host immune response against *S. sobrinus* infection as an immunosuppresive protein (95). However, this enolase function has not been yet investigated in *S. suis* and further investigation is needed to confirm it.

The enolase from microorganisms such as *Saccharomyces cerevisiae* (39) and *Lactobacillus rhamnosus* (72) has also been described as a heat shock protein. A heat shock protein is a specific protein expressed and/or secreted by cells that permit its adaptation to extreme environments such as high temperatures (66). In our effort to characterize SsEno we have also described that SsEno is also heat shock protein (Annex 3, Fig 1). In *S. suis*, it can be proposed that upregulation of enolase is involved in thermal tolerance and growth control of bacteria. These results showed in this work confirm that α-enolase should not be more considered as a housekeeping enzyme since its expression varies depending on the pathophysiological, metabolic or developmental conditions of cells. Furthermore, these results confirm that SsEno is a multifunctional protein that can participate in the different steps of the pathogenesis of the infection caused by this pathogen depending on the function it develops in each moment.

To be a fibronectin-plasminogen and IgG-binding protein, SsEno has to be present on the surface of *S. suis*. In fact, we have shown by several methods that SsEno is present in the surface of all the described *S. suis* serotypes (Annex 2, Fig 1) corroborating its role as possible adhesin. Up to now, three types of streptococcal fibronectin-binding adhesins have been identified (14). The first ones are the MSCRAMM (microbial surface component recognizing adhesive matrix molecules) proteins, which are the most abundant type (more than 90% of the bacterial fibronectin-binding proteins) (70). This adhesin contains a signal sequence at N-terminal and a LPXTG motif at C-terminal domain which is recognized by a transpeptidase called sortase (69). As explained before, sortase is a multifunctional protease-transpeptidase that specifically cleaves the LPXTG sequence in a protein and catalyzes the transfer of the processed protein to the free amino group of pentaglycine cross-bridges in peptidoglycan. The second type of fibronectin-binding protein is the choline-binding proteins (32). This sort of proteins lacks the LPXTG motif but contained carboxy-terminal choline-binding repeats that interact with the phosphorylcholine of the lipoteichoic acid of Gram-positive bacteria. Finally, the last type described is the new denominated anchorless-proteins (14). These types of protein lack the conventional leader sequence, the LPXTG motif and the choline-binding sequences. It is important to note that up to now, the mechanism used for these last type of proteins to reach the surface of bacteria is still unknown. We have demonstrated (Annex 8, Fig. 2) that the ability of a *S. suis* mutant strain lacking the srtA to interact with fibronectin is reduced compared to the wild-type strain. However, it still possesses more than 70% of its fibronectin-binding activity, indicating that, although surface proteins anchored by sortase A are required for a normal level of bacterial binding, other proteins different than MSCRAMM are the principal adhesins involved in the interaction between *S. suis* and fibronectin. The amino acid sequence analysis of SsEno shows that this protein does not possess an N-terminal signal sequence and an anchor carboxyl terminus LPXTG motif, the crucial domains for translocation through the cell wall and for anchoring surface proteins to the Gram-positive bacterial cell wall (26). Furthermore, repeats typical of choline-binding proteins such as those described for the choline-binding protein of *S. pneumoniae* (102) are also not present on SsEno. All these features classify SsEno as one of the new anchorless-type adhesins and confirm our previous results indicating that the major *S. suis* fibronectin-binding proteins do not belong to the MSCRAMM family of proteins. In addition to its surface localisation, SsEno is also present in all the other subcellular compartiments of the bacteria and it is also secreted to the medium. It is largely unknown how glycolytic enzymes like enolase and GAPDH that lack a signal peptide required for secretion and peptidoglycan-anchoring motifs are transported to and associated with bacterial surface structures or which factors influence this process. It also remains open as to whether these proteins are secreted across the bacterial cell membrane by the general secretory (Sec) pathway. One study suggested that the secretion of the *Listeria monocytogenes* enolase, which also binds plasminogen, is SecA2-dependent (52). Another

study speculated that surface GADPH and enolase do not come from inside the bacteria but are 'scavenged' from other cells that have undergone programmed cell death through allolysis (31). However, further research is necesary to finally understand this mystery.

As mentioned above, the pathogenesis of *S. suis* infection is poorly understood at present. Once in the bloodstream, *S. suis* resists phagocytosis and killing by neutrophils and monocytes (8, 12, 78). In the event that *S. suis* fails to cause acute fatal septicemia, bacteria are able to reach the CNS (22) via different mechanisms that are only partially elucidated and produce meningitis. One of the mechanisms proposed to cross the BBB is the adhesion to, and posterior invasion of the brain microvascular endothelial cells (BMEC) (4, 13, 93) and/or choroid plexus epithelial cells (88, 89). As fibronectin-binding proteins have been described as important virulence factors that participate in adhesion to and invasion of host cells for other pathogens (14), the third objective of this work was to determine the role of SsEno in the pathogenesis of the infection caused by *S. suis*. SsEno is the major *S. suis* fibronectin-binding protein described up to now, so we proposed that the interactions of SsEno to fibronectin may be an important key in this mechanism. In addition, this hypothesis is corrobored by the fact that *S. suis* adhesion to and intracellular invasion of PBMEC increases in more than 500% and 700% respectively when bacteria are precoated with fibronectin (94), indicating that this *S. suis*-fibronectin adhesion is an important step for the development of the infection. An important tool to demonstrate the role of a fibronectin-binding protein (SsEno in the case of *S. suis*) in the pathogenesis of the infection caused by this pathogen is the construction of a knock-out mutant. However, as SsEno is an essential enzyme that participates in the glycolysis and gluconeogenesis of the bacteria (1), the production of a knock-out mutant for this protein was not possible. Another method could be a knock-out mutant for the export system of this protein to the bacterial surface however, as this mechanism is still unknown, this approach was also not possible. The alternative method that we used to determine the role of this adhesin in the adhesion to and invasion to host cells was by blocking the SsEno function with specific antibodies againts this protein. Our results show that the "inactivation" of SsEno with specific antibodies against this adhesin significatively decreases the adhesion to and the invasion of *S. suis* to these host cells corroborating that the *S. suis* fibronectin-adhesion is an important step of the infection and demonstrating that the adhesion of *S. suis* to fibronectin via its SsEno is important for this process.

On the other hand, although the adhesion to and invasion of *S. suis* to PBMEC was not increased by the addition of exogenous plasminogen, a certain role of the plasminogen-binding activity of SsEno in the pathogenesis of the infection caused by *S. suis* cannot be ruled out. It has been previously demonstrated that plasminogen adhered to *S. suis* is able to acquire plasmin activity when it is incubated with BMEC (42). As a consequence, bacterium is able to hydrolyse ECM

proteins, such as fibronectin, and destabilise the cells forming barriers. Once bacteria degrade the ECM, cells loss their stability and start to die by anoikis (28). Anoikis is a type of cell death that produces "holes" in the barriers. These "holes" can be used by pathogens to facilitate their penetration through biological membranes (28). To demonstrate this hypothesis, future work using, for example, a transwell system could be done (Fig. 2). This system permits to determine if the presence of a substance in the medium (in our case plasminogen) enhance bacteria to cross a monolayer of BMEC. This method consists in, as it is shown in Fig 2, two compartments separated by a porous membrane where cells, in this case BMEC, grow. Once cells form a monolayer, both compartments are physicaly separated implicating that if you put bacteria in the upper compartment and after a period of time you recover bacteria from the inner compartment is because bacteria have crossed the cell monolayer intracellularly (invading cells) or intercellularly (by broking the cell junctions). The transwell method also permits to know which method is using bacteria to cross the cell monolayer by measuring the transendothelial electrical resistance (TEER). If bacteria cross the cell monolayers by invading cells, cells monolayer integrity is not disturbed and the TEER will not change. In contrast if bacteria broke the junctions between cells, TEER will be modified.

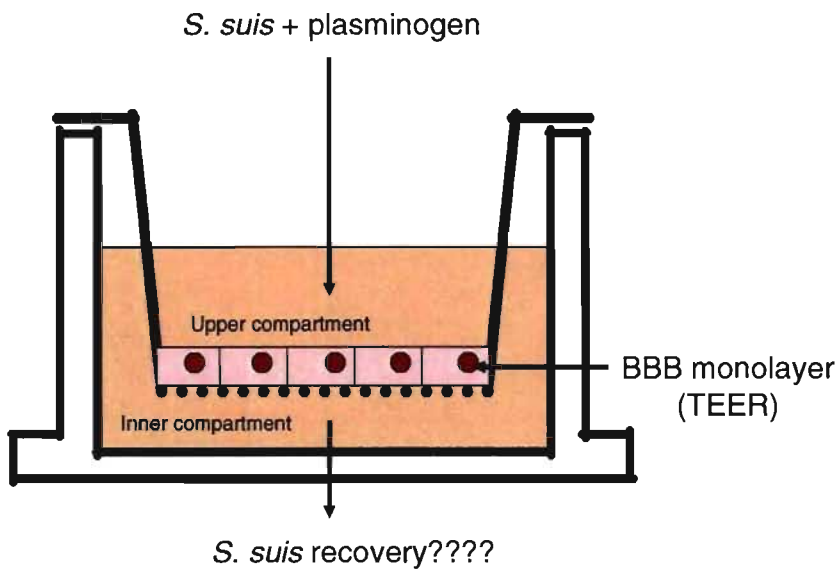

Fig 2. **Transwell system.** Upper and inner compartments are separated by a porous membrane where BMEC grow to a confluent monolayer of cells producing a stable TEER. *S. suis* with or without plasminogen can be introduced in the upper compartment and invasion can be measured by recovering or not bacteria from the inner compartment. Changes in TEER indicates if the bacteria cross the monolayer by invasion or by broking the junctions between cells.

As a result of its critical role in the infection process, bacterial adherence to host tissues represents a potential target for the development of new antimicrobial agents. As explained before, different types of vaccine against *S. suis* infections have been developed or are currently under investigation. At present, bacterins (commercial and autogenous) have been used in the field to prevent *S. suis* disease. However, disappointed results are usually obtained since these vaccines do not induce high levels of antibodies and they are serotype-specific (37). Recently, we have demonstrated that a surface expressed protein (Sao) confers protection against an experimental infection in mice and pigs (53)(Annex 7). However, this protection was not complete, which indicates that other proteins should probably be combined with Sao to optimize the protective capacity.

Agents that can block adherence, such as specific antibodies, may prove to be effective in combating infectious disease. In fact, fibronectin-binding proteins have been suggested as potential vaccine targets for preventing bacterial infections because antibodies directed against such adhesins may prevent bacterial attachment and inhibit colonization (100). As SsEno is the major fibronectin-binding protein described for *S. suis* up to now, the last objective of this work was to test its potential as vaccine candidate in a septicemia and meningitis model in the mouse (22). SsEno has been chosen as a good vaccine candidate because: i) it is present in the surface of all the 35 different serotypes described up to day for *S. suis* (Annex 2, Fig 1) , ii) it participates in the adhesion to and invasion of *S. suis* to the host cells, iii) it is a highly conserved protein without antigenic variation (24), and finally iv) it is a highly immunogenic protein that elicits elevated antibody levels in sera from pigs. This is not the first time that an enolase has been tested as vaccine candidate to protect hosts from different organism. It has been demonstrated that anti-enolase antibodies are able to protect mice against *Plasmodium falciparum* infection (65). However, recombinant enolase from *Candida albicans* induced only a modest protection against disseminated candidiasis (61). To our knowledge, this is the first time that a protection test against streptococci using enolase as antigen to elicit an immune response has been done. The results obtained in this work show that, although the good immune response elicited by SsEno [all SsEno-specific IgG isotypes (IgG1, IgG2a, IgG2b and IgG3) were induced], these antibodies are not able to protect mice in an experimental infection with *S. suis*. As it has been shown that antibodies derived from hyperimmune sera against whole bacteria can protect mice against infection (11) and induce bacteria killing (8) we hypothesized that this lack of protection is due to the fact that anti-SsEno antibodies are not opsonic, permitting bacteria to reproduce in high numbers and cause disease. To verify this hypothesis we evaluated the capacity of the purified anti-SsEno IgGs to opsonise bacteria and promote their killing by the murine phagocytes. The results obtained in this work showed that, although SsEno elicits an important

immune response in convalescent pigs and immunized mice, the produced anti-SsEno IgGs are not able to induce the *S. suis* killing by mouse phagocytes being this response inadequate for protection against *S. suis* infections.

In conclusion, although we have not found the "good" vaccine candidate against *S. suis* infections, this work is important because it provides new advances in the study of the infectious process produced by *S. suis* and permits a better comprehension of the pathogenesis of the infection caused by this important pig and human pathogen (Fig. 3). In fact, we have described for the first time that the pathogen *S. suis* is able to adhere to fibronectin and collagen, the two main components of the ECM. In addition, we have identified new genes that participate in the adhesion of *S. suis* to collagen. Moreover, we have also identified and characterized a new *S. suis* multifunctional adhesin with fibronectin-plasminogen and IgG-binding activities that participates in the adhesion to and invasion of this pathogen to the endothelial cells forming the BBB which can permit bacteria to cross this host barrier to the CNS and produce meningitis. It is important to remark that this is the first time that the fibronectin and the IgG-binding activities of a bacterial α-enolase are described. Finally, we have also described that SsEno is a heat shock protein that could be involved in thermal tolerance and growth control of bacteria.

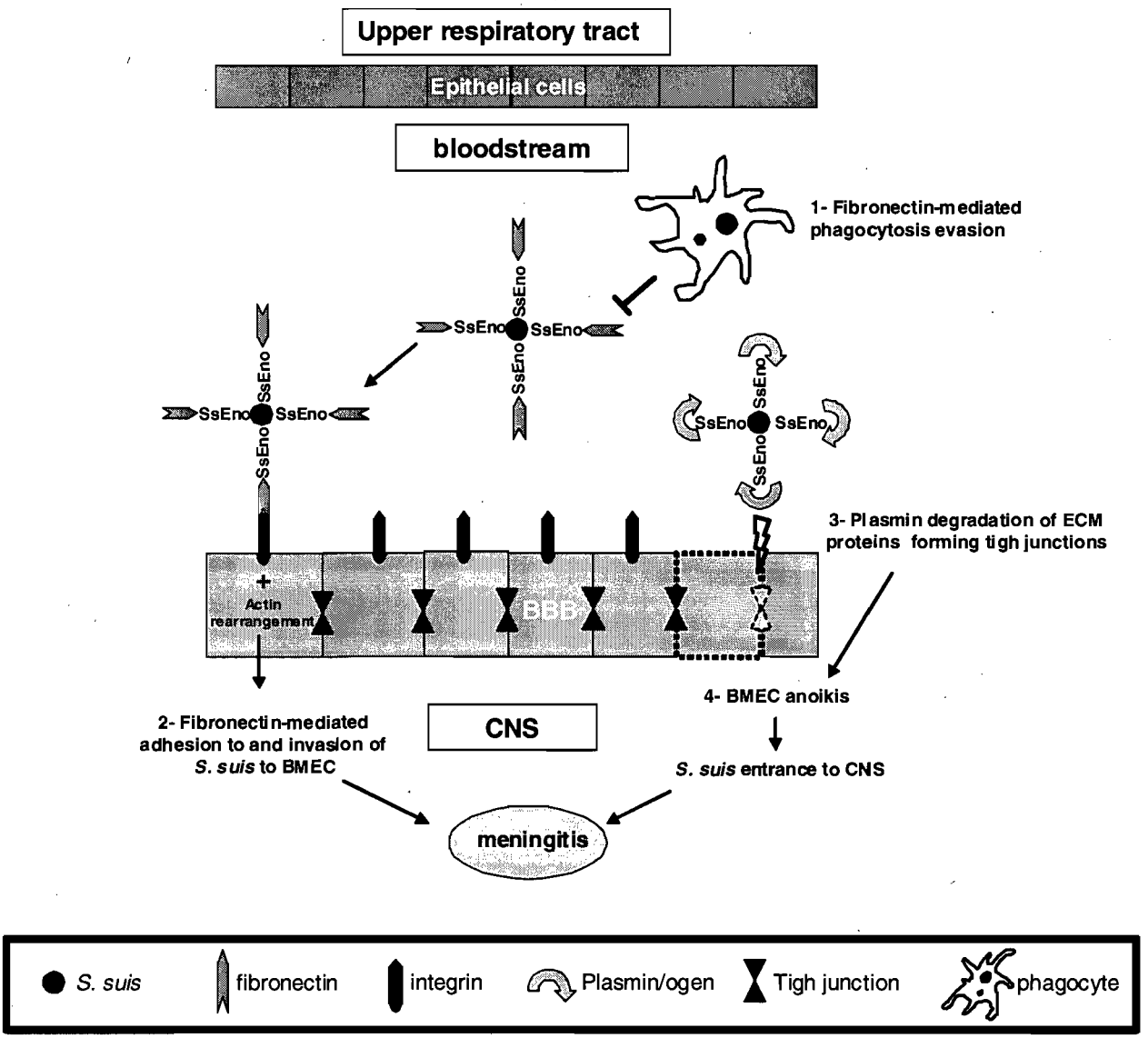

Fig 3. Potential roles of SsEno in the pathogenesis of the infection caused by *S. suis*. The new described multifunctional SsEno adhesin can participate in the pathogenesis of the infection caused by *S. suis* by several ways. As fibronectin-binding protein, this protein can permit the evasion of *S. suis* from the immune system cells since fibronectin-coated *S. suis* can not be recognised by these cells as an exogen particle and, as consequence, it is not phagocytosed and killed by these cells (1). In addition, the SsEno adhered fibronectin can also be recognised by the integrins on the surface of several cells such as the BMEC, producing the activation of its cytoskeletton and permitting the invasion of this pathogen to these cells (2). This mechanism is considered as one of the ways by which bacteria is able to cross some host barriers such as the BBB. Finally, the plasminogen-binding activity of this protein can permit bacteria to adhere to plasminogen that, in presence of plasminogen activators normally secreted by host cells, is converted in the protease plasmin. This protease is able to degrade the ECM-forming junctions between cells (3) provoking anoikis (4) and as consequence, holes in the barriers by where bacteria can traverse to the CNS (5).

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V. GENERAL CONCLUSIONS

- *Streptococcus suis* adhere to some of the components of the ECM such as plasma and cellular fibronectin and collagen types I, III and V. However, although *S. suis* can not directly bind to collagen type IV, this pathogen can use plasma fibronectin as a bridge to permit this adhesion. This mechanism can be considered as a new mechanism of colonisation and evasion.

- The adhesins involved in these adhesions are mostly proteins. However, although most of the bacterial fibronectin-binding proteins are MSCRAMMs, in the case of *S. suis* these type of adhesins are responsible of less than 30 % of these adhesions. The other 70 % of adhesion can be done by choline-binding proteins or by the new denominated anchorless-proteins.

- The major *S. suis* fibronectin-binding protein described up to day is the surface □-enolase. As expected, this fibronectin-binding protein is not an MSCRAMM but an anchorless-binding adhesion.

- SsEno adheres to human and porcine fibronectin indicating that this protein could enhance the infection of this pathogen in both hosts

- In addition to its fibronectin-binding activity, SsEno is also responsible of the adhesion of *S. suis* to plasminogen and to IgG from human, porcine and chicken origin.

- SsEno is also a HSP confirming that this protein can not be more considered as a housekeeping enzyme.

- SsEno participates in the adhesion to and invasion of brain microvascular endothelial cells (BMEC), the cells forming the BBB, which implies that this protein can be considered as a new virulence factor that participate in the pathogenesis of the infection caused by *S. suis*

- Although SsEno is a highly conserved immunogenic protein present in all the described serotypes of *S. suis*, this protein does not develop an effective immune response able to protect host from *S. suis* infections. These results suggest that antibodies against other *S. suis* components are the responsible of the solid immunity developed in pigs that have undergone infections caused by this pathogen and that new efforts have to be done to finally developed the effective vaccine.

- Despite the fact that some of the steps involved in the pathogenesis of *S. suis* meningitis remains to be solved, our findings in this work suggest that adhesion of *S. suis*, via SsEno, to the fibronectin present in the cells forming BBB represent a key step in the development of the disease and provide a little advance in the comprehension of the pathogenesis of the infection caused by this important pig and human pathogen.

VI. ANNEX

ANNEX 1

Purification of plasma porcine fibronectin

One of the main objectives of this work was to study the capacity of the swine pathogen *S. suis* to bind to some of the most important ECM proteins. One of the main results of this work is that *S. suis* is able to specifically bind to fibronectin. As explained before, the adhesion of bacteria to fibronectin helps bacteria in the pathogenesis of the infection caused by these pathogens (2). In one hand, bacteria can use their fibronectin-binding proteins to bind to soluble fibronectin facilitating their evasion from host immune surveillance. On the other, since fibronectin binds to membrane cell receptor proteins called integrins, fibronectin-coated bacteria are able to bind to these cell receptors permitting the activation of the cell cytoskeleton and, as consequence, the posterior invasion to these cells. Both mechanisms help bacteria to survive in the host and to develop an infection (2).

Although *S. suis* is mainly a swine pathogen, the fibronectin used in the first objective of this was a commercial human plasma fibronectin. However, to test the capacity of SsEno to bind to fibronectin we decided to test both, the human and the porcine plasma fibronectin. As this porcine fibronectin is not commercially available, a simple protocol for its purification from porcine serum was designed.

The first step consisted in a pre-purification of fibronectin from porcine plasma using the high affinity of this protein for gelatin (1) (Fig. 1). Briefly, a nitrocellulose membrane was coated with porcine gelatin for 2 hours before its incubation with porcine blood plasma. Blood plasma is the liquid component of blood, in which the blood cells are suspended. It makes up about 55% of total blood volume. It is composed of mostly water (90% by volume), and contains dissolved proteins including fibronectin among other components. Plasma fibronectin is synthesized and secreted into the bloodstream by hepatocytes and found at 300-500 μg per ml in plasma (3). The porcine blood plasma used in this work was prepared simply by spinning a tube of fresh blood until the blood cells fall to the bottom of the tube. The blood plasma was then recovered and kept at -20°C until its utilization.

As explained, porcine blood serum was incubated for 2 hours with the gelatin coated nitrocellulose membrane. After that, membrane was washed three times with PBS to eliminate all the proteins that did not bind to gelatin and the gelatin attached proteins (including fibronectin) were eluted using Urea 8M.

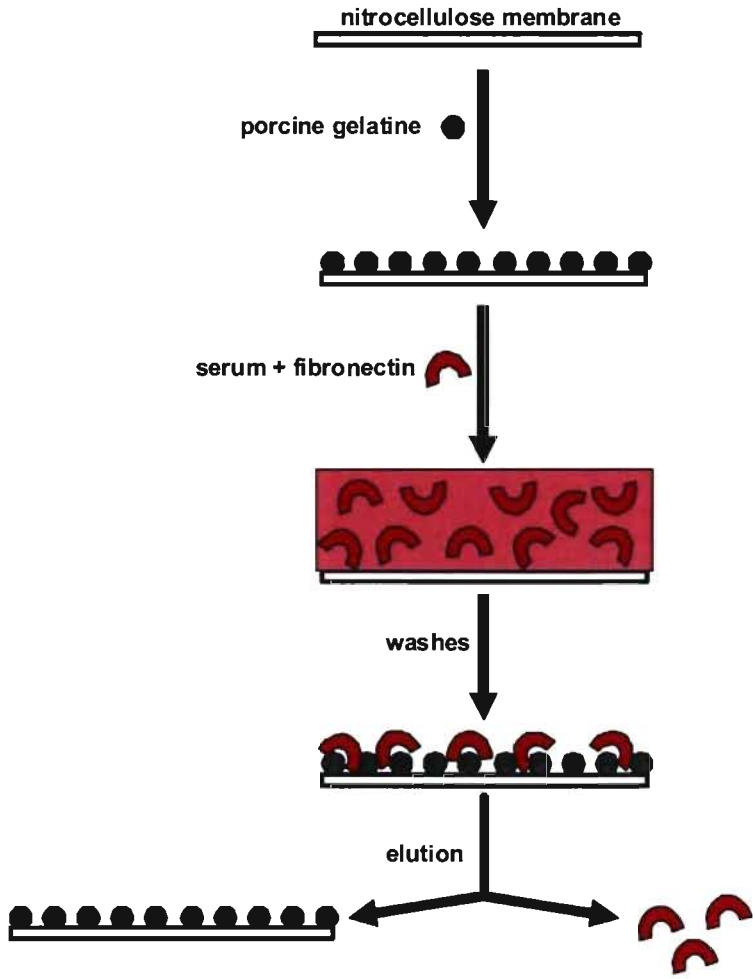

Fig 1. Porcine plasma fibronectin purification. Porcine plasma fibronectin was purified from porcine fresh blood using gelatine-coated nitrocellulose membranes. After two hours of incubation, non attached proteins were washed and fibronectin was recovered by elution with Urea 8M.

After this first step, different proteins, including the 250 kDa fibronectin protein, were obtained (Fig. 2)

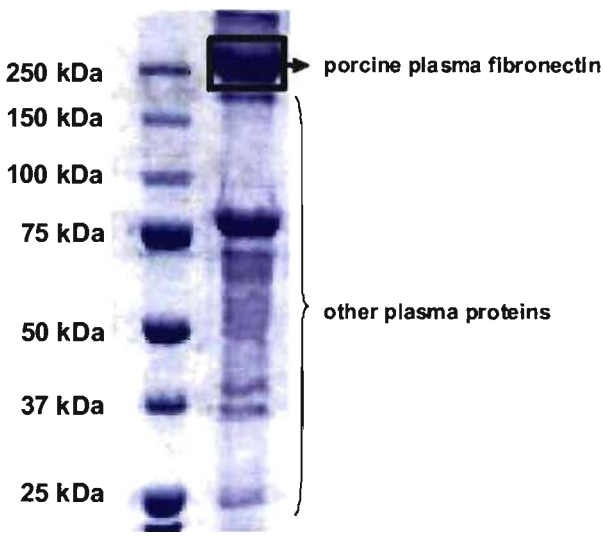

Fig 2. Proteins recovered after the first step of the purification of porcine plasma fibronectin. Plasma fibronectin was incubated with gelatin-coated nitrocellulose membranes and the proteins with high affinity for gelatin were eluted using Urea 8M, separated with 10% SDS gel and stained with coomasie blue.

To purify fibronectin from all the other proteins obtained in step 1, we performed a High-performance liquid chromatography (HPLC). HPLC is a form of column chromatography used frequently to separate, identify, and quantify compounds. This method utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, molecules being analyzed, and the solvent(s) used. The binding buffer used was 20 mM Tris HCl, pH 8. The elution buffer used was 20 mM Tris-HCl, 1 M NaCl. We tried different elution buffer pHs and different times of elution to find the best protocol. The different pH used were pH 4, pH 7 and finally pH 10. We also tried as elution times 15, 30 and 45 minutes. The protocol that worked best was that with pH 7 and 45 minutes of elution (Fig. 3). In these conditions we obtained 2 peaks. The first one did not contain fibronectin whereas the second one contained high pure fibronectin (Fig.3)

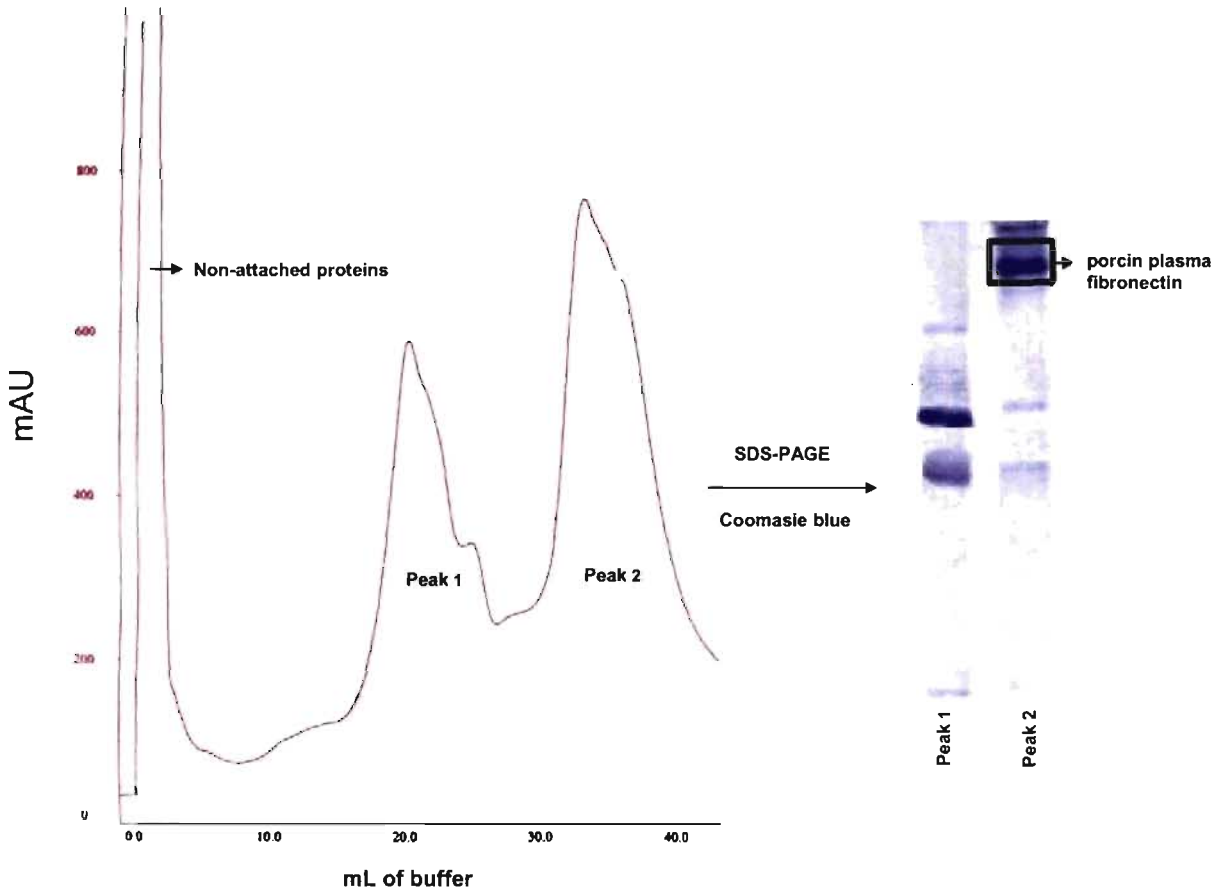

Fig 3. Porcine fibronectin purification. The recovered proteins of the step 1 were separated using HPLC. With an elution time of 45 minutes and an elution buffer pH 7 two peaks were obtained. By SDS-PAGE and coomasie blue staining we concluded that the second peak contains a high pure (≥ 99% of purity) porcine plasma fibronectin.

The obtained purified porcine plasma fibronectin was used to test the adhesion of SsEno to this fibronectin by Western blot. Results obtained showed in Fig 4 demonstrate that in addition to its adhesion to human fibronectin, SsEno also adheres to porcine fibronectin, indicating that *S. suis* can use the fibronectin-binding activity of SsEno as a mechanism to colonize, adhere to and invade cells from both hosts, human and swine.

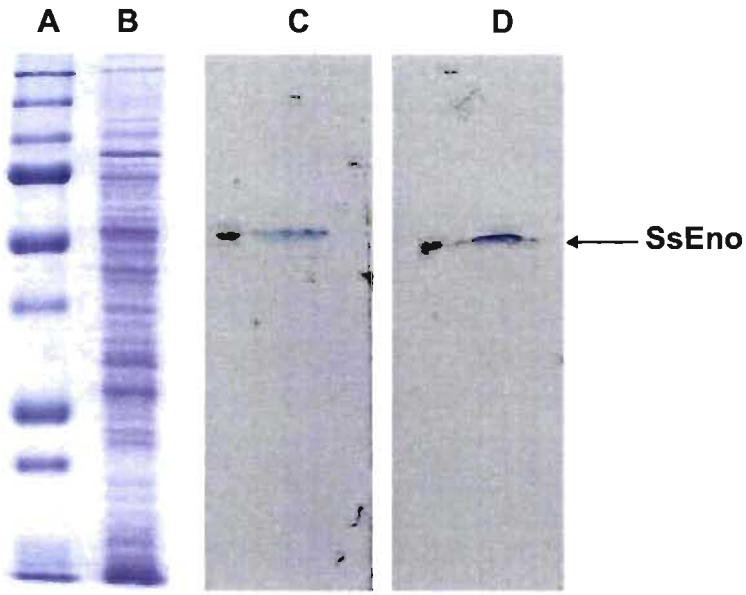

Fig 4. SsEno is able to adhere to human and porcin fibronectin. *S. suis* proteins, including SsEno (arrow) were determined by SDS-PAGE (12.5%) followed by Coomasie blue staining (B). Proteins were transferred to a nitrocellulose membrane and the fibronectin-binding activity of SsEno was determined by Far western blot using commercial human (C) and porcine plasma purified (D) fibronectin. Molecular markers (A) were 250, 150, 100, 75, 50, 37, 25 and 17 kDa.

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ANNEX 2

SsEno is present in all the 35 described serotypes for *S. suis*

Streptococcus suis is a major porcine pathogen worldwide that, in addition, can be transmitted to human beings by close contact with sick or carrier pigs (5). *S suis* causes meningitis, septicaemia and septic shock among other diseases in both pigs and human (5). Moreover, it can also produce the death (5). Human infection with *S suis* occurs mainly among certain risk groups that have frequent exposure to pigs or pork (5). Outbreaks of human *S suis* infection are uncommon, although several outbreaks have occurred in Asia in recent years (7). In July, 2005, the largest outbreak of human *S. suis* infection occurred in Sichuan province, China, where 204 people were infected and 38 of them died. There have been more than 400 cases of human *S. suis* infection worldwide, most of which have occurred in China, Thailand, and the Netherlands, and these infections have led more than 70 deaths (7).

Among the 35 serotypes described, the serotype 2 has always been considered the most virulent and the most frequently isolated serotype from diseased animals (3). However and as explained in this work, the situation may be different depending the geographical location and also, throughout the time. The percentage of *S. suis* serotype 2 strains isolated from diseased animals in North America remained relatively low compared with those reported in some European countries, such as France, Italy and Spain, where most of isolates recovered from diseased animals belong to serotype 2 (1, 9). In this regard, it may be hypothesized that European and North American serotype 2 strains of *S. suis* possess a different virulence potential (3). In addition, under specific circumstances, some strains belonging to other serotypes of *S. suis* appear highly virulent, as it is the case for serotype 14 in UK (4), serotypes 1/2 (unpublished observations M.Gottschalk) and 5 (2) in Canada and serotype 9 in central Europe (9). In contrast of what occurs in swine, except for two human *S. suis* infection cases caused by serotype 1 (although not serologically confirmed), two cases of septicemia caused by serotype 14 and one case caused by serotype 4, all other human *S. suis* infections are attributed to serotype 2 (6, 8).

At the present time, vaccination of pigs to prevent swine infections but also human infections is generally carried out with autogenous bacterins but results are equivocal. Up to day, several *S. suis* virulence factors have been tested as vaccines candidates, however, most of them protected only against a homologous strain of this pathogen.

We have demonstrated in this work that SsEno is a *S. suis* multifunctional adhesin with fibronectin-binding activity. It has been demonstrated that agents that can block adherence, such as specific antibodies, may prove to be effective in combating infectious disease. In fact, fibronectin-binding proteins have been suggested as potential vaccine targets for preventing bacterial infections because antibodies directed against such adhesins may prevent bacterial attachment and inhibit

colonization (10). As SsEno is an important fibronectin-binding protein of *S. suis*, we can hypothesise that it can be an interesting candidate to be used as a vaccine against *S. suis* infections. However, to be a good candidate for a vaccine, SsEno had to be present at the surface of all the serotypes described for this pathogen.

To test the presence of SsEno in the surface of all the described *S. suis* serotypes, the reference strain of each serotype was grown overnight onto sheep blood agar plates at 37°C and isolated colonies used as inocula for 5 ml of Todd-Hewitt broth (THB; Difco Laboratories), which was incubated for 8 h at 37°C with agitation. Working cultures were prepared by transferring 10 µl of 1/1000 dilutions of 8-h cultures into 30 ml of THB which was incubated for 16 h at 37°C with agitation. Stationary phase bacteria were washed twice in PBS (pH 7.3). Bacterial pellet was then resuspended in THB until an OD600 nm value of 0.4 was achieved, which corresponded to 5 x 10^8 CFU/ml. Twenty-five microlitres of this dilution was blotted in a nitrocellulose membrane and air-dried. The membrane was then blocked in Tris buffered saline (TBS) containing 2% casein for 1 h with agitation, then incubated 2 h in the specific rabbit anti-SsEno antibodies produced as described in this work. The membrane was then washed in TBS, and incubated 1 h with the anti-rabbit/horseradish peroxydase conjugate (Jackson Immunoresearch), diluted 1/2000 in TBS. The membranes were then washed again in TBS before the addition of the 4-chloronaphtol/hydrogen peroxyde based revelator for 15 min, washed in water and dried.

The results obtained demonstrated that this protein was present in the surface of all the reference strains of the 35 *S. suis* described serotypes (Fig. 1) corroborating that this protein was an interesting candidate to be used as an antigen in a protection assay against a *S. suis* infection.

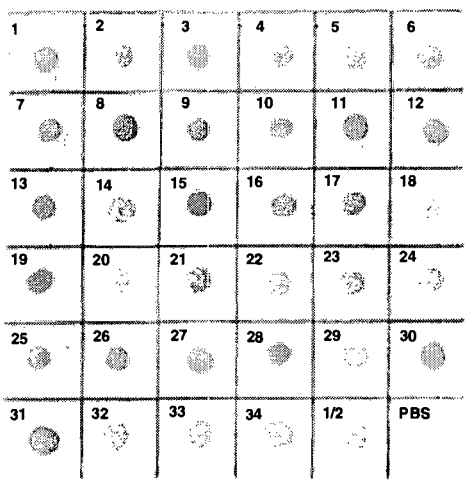

Fig. 1. SsEno is present at the surface of all the described *S. suis* serotypes. The same concentration of each one of the reference strains of the 35 serotypes of *S. suis* were grown in THB before being blotted in a nitrocellulose membrane. The detection of SsEno was done with rabbit anti-SsEno antibodies. Each square represent a serotype of *S. suis*. PBS was used as a negative control.

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ANNEX 3

Characterisation of SsEno as a heat shock protein (HSP)

In adapting to extreme environments such as high temperature or glucose deprivation, cells often secrete or express specific proteins known as heat-shock proteins (HSPs) (5). It has been demonstrated that vascular endothelial cells in a similarly stressful situation such as chronic hypoxia respond by upregulating the expression of a unique set of five cell-associated stress proteins (Mr 34, 36, 39, 47 and 57) in a time- and oxygen concentration-dependent manner (3). The 52-kDa protein has been identified as α-enolase. This was the first time that an α-enolase was identified as a HSP. It is proposed that in a hypoxic situation, upregulation of α-enolase may provide protection to cells by increasing anaerobic metabolism (1). Later, it has been demonstrated that in *Saccharomyces cerevisiae* several HSPs were also induced at elevated temperatures. One of these HSPs, HSP48, was also identified as α-enolase (4). Finally, it has been demonstrated that the HtrA surface protease in gram-positive bacteria is involved in the processing and maturation of extracellular proteins and degradation of abnormal or misfolded proteins (2). Inactivation of htrA in *S. mutans* has been shown to affect the tolerance to thermal and environmental stress and to reduce virulence (2). This inactivation also resulted in a reduced ability to withstand exposure to low and high temperatures, low pH, and oxidative and DNA damaging agents (2). In addition, the htrA mutation affected surface expression of several extracellular proteins including enolase and glyceraldehyde-3-phosphate dehydrogenease, two glycolytic enzymes that are known to be present on the streptococcal cell surface (2).

As we have identify the *S. suis* enolase (SsEno), we investigated if a change in growth temperature could also influence the production of SsEno as it has been described in the other works described before (2, 4). The ability of strain 166′ of *S. suis* serotype 2 to respond to a heat stress via SsEno was assessed by growing the organism at 37°C in Todd-Hewitt broth (THB) and then shifting the temperature to 42°C for 2 h. Optical density was recorded in order to measure cell growth. One ml of each culture was centrifuged for 5 min at 12 000×g and the supernatant was discarded. The same quantity of protein for the stressed and unstressed cells was subjected to SDS-PAGE using a 10% gel. The identity of the SsEno as hsp was confirmed by immunoblotting using the specific polyclonal antibody against SsEno.

The results obtained showed that after 2 h growth at 42°C compared with the control culture grown at 37°C an increase in expression of the 52-kDa protein, determined as SsEno by Western blot using the purified rabbit anti-SsEno IgG, was observed as expected for a Hsp (Figure 1).

Regarding this results we can conclude that, in addition to a fibronectin and plasminogen-binding protein, this enzyme has also heat shock activity that, as it has been demonstrated for other pathogens, could be involved in the thermal tolerance and the growth control in this pathogen. However, further research is necessary to confirm this hypothesis.

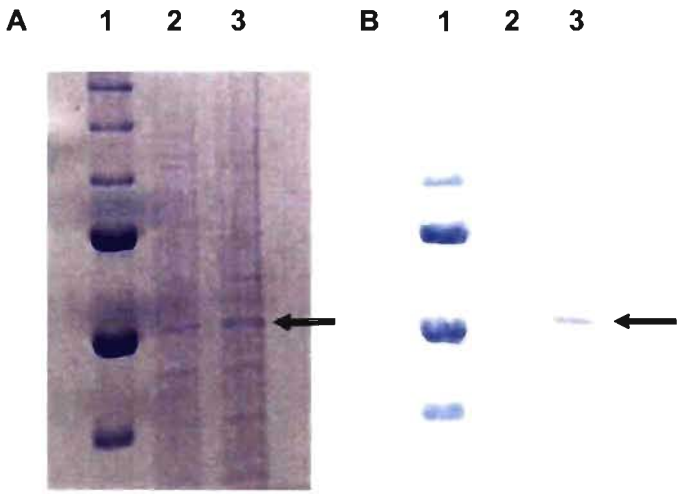

Fig. 1. SsEno is a heat shock protein. Cells were grown at 37°C and the temperature shifted to 42°C for 2 h. Coomassie blue-stained gel (A) and nitrocellulose blot incubated with polyclonal anti-SsEno antibody (B) showed the heat shock activity of SsEno. Lane 1, molecular marker (kDa); lane 2, cells kept at 37°C; lane 3, cells shifted to 42°C.

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ANNEX 4

Characterisation of SsEno as an IgG-binding protein

The expression of bacterial surface proteins that interact nonspecifically with immunoglobulins (Igs) from different mammalian species has been described for many microorganisms (2). Nonimmune binding to Igs is generally mediated by the Fc fragment of the Ig molecules (2). However, few of these proteins can also bind the F(ab')2 fragment through an alternative binding pathway (2). The consistent presence of these Ig-binding proteins in many pathogenic bacteria suggests that these molecules might be required for bacterial survival during the infection process.

Based on their binding activities with IgG from various host species, different types of IgG-binding proteins have been proposed. Well-known examples of such proteins are protein A from *S. aureus* (3) and protein G (6) from group C and G streptococci, both of which preferentially bind IgG. Some group A streptococcal proteins have been purified and extensively characterized: protein Arp (1), which preferentially binds IgA; protein H (4), which binds IgG; and protein Sir (7), which binds IgA and IgG. These various Ig-binding proteins are valuable as immunochemical tools and as model systems with which to study the interactions between Ig and specific receptors. In addition, some reports have demonstrated an association between expression of Ig-binding proteins by group A streptococci and the ability of these microorganisms to elude host defenses (5).

Some Ig-binding proteins were shown to bind other plasma proteins, such as albumin and fibronectin (5). In fact, it has been described that fibronectin-binding protein I (SfbI) of *S. pyogenes*, a main adhesin and invasin, also binds to human IgG in a nonimmune fashion through the Fc component (5). This binding affected Fc-mediated phagocytosis by macrophages and antibody-dependent cell-mediated cytotoxicity. SfbI was also reactive with mouse, rabbit, pig, and horse IgG (5).

As SsEno has been described in this work as an important *S. suis* fibronectin-binding protein, the ability of this protein to bind to IgG was also tested. We tested IgG from different species, the two main host of *S. suis* (pig and human) and chicken IgG (IgY).

Results showed in Fig 1 demonstrate that, in addition to its fibronectin- and plasminogen-binding and heat-shock activities, SsEno is also able to adhere to human, porcine and chicken IgGs. In fact, this is the first time that the IgY-binding capacity of a bacterial protein is demonstrated.

The chicken immune system has been studied for many years and these studies have contributed substantially to our understanding of the fundamental concepts of immunology and the development of different immunoglobulin classes. It is thus surprising that only a small fraction of the antibodies presently used in laboratories are of avian origin. A laying hen produces more yolk antibodies than a rabbit can produce during the same time period, and the animal care costs are

lower for the chicken compared to the rabbit. Chicken antibodies offer many advantages to the traditional mammalian antibodies when used for the detection of mammalian antigen. Due to the evolutionary difference chicken IgY will react with more epitopes on a mammalian antigen, which will give an amplification of the signal. Chicken antibodies can also be used to avoid interference in immunological assays caused by the human complement system, rheumatoid factors, human anti-mouse IgG antibodies or human and bacterial Fc-receptors. The antibodies can be purified in large amounts from egg yolk, making laying hens highly efficient producers of polyclonal antibodies. However, up to day it does not exist a rapid and simple protocol for their purification. Our results open the door for a new method for IgY purification using SsEno as a ligand.

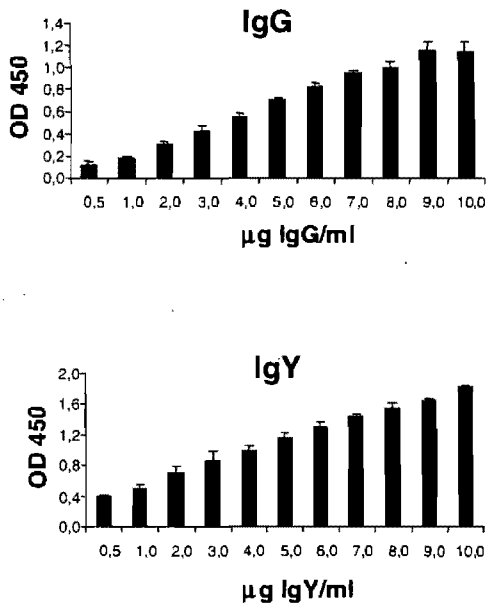

Fig.1. Representative ELISA analysis for the binding to 5 ug/ml SsEno binding to immobilized human IgG and IgY. Similar results were obtained with porcine IgG. Binding was evaluated after 2 h. Data are expressed as means ± standard deviations of at least three experiments performed in triplicate.

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ANNEX 5

Characterisation of SsEno as a collagen-binding protein

In the first objective of this work we have observed a cross-inhibition with soluble fibronectin and collagen in the ECM binding assay, indicating that fibronectin and collagen binding domains exist on the same or nearby bacterial surface adhesins. To test if SsEno is one of the proteins responsible for this cross-inhibition between both proteins an ELISA binding assay between collagen and SsEno was done to test if SsEno is also responsible of the collagen-binding activity of *S. suis*.

Purified rSsEno binding to immobilized collagen (Sigma) was determined by ELISA. Briefly, Maxisorp® flat-bottom microtiter 96-well plates (Nunc, VWR, Mississauga, ON, Canada) were coated with 100 µl of serial dilutions collagen in 0.1 M carbonate coating buffer (0.15% [wt/vol] Na2CO3, 0.1% [wt/vol] MgCl2 - 6H20, 0.3% [wt/vol] NaHCO3 [pH 9.6]) and incubated overnight at 4°C. Casein-coated wells served as a control for non-specific adhesion of rSsEno. Fibronectin and plasminogen coated wells served as a positive control for specific adhesion (Fig. 1 A and B). The plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST, pH 7.3), and 200 µl of 3% (w/v) non-fat dry milk in PBST was added to each well to prevent non-specific binding. After 1 h at 37°C, the wells were washed three times with PBST. Next, 100 µl of 5 µg ml-1 of rSsEno was added and the plates were incubated for 2 h at 37°C. After several washes with PBST, optimally diluted rabbit anti-SsEno IgG were added to each well and the plates were incubated for 1h at 37°C. The wells were washed three times with PBST and specific horseradish peroxidase-labelled IgG (Jackson Immunoresearch Laboratories) was added. Plates were then incubated for 1 h at 37°C with the secondary antibody and 3,3',5,5'-tetramethylbenzidine (Zymed, San Francisco, CA, USA) was used as the enzyme substrate according to the manufacturer's instructions. The reactions were stopped by adding 25 µl of H2SO4 (1N) and were read at 450 nm using a microplate reader (Uvmax; Molecular Devices, Menlo Park, CA, USA).

Results showed that SsEno does not possess collagen-binding activity (Fig. 1 C) and that, as consequence, it is not the responsible of the cross-inhibition between fibronectin and collagen. These results also indicate that other collagen-binding proteins are responsible of the adhesion of *S. suis* to collagen.

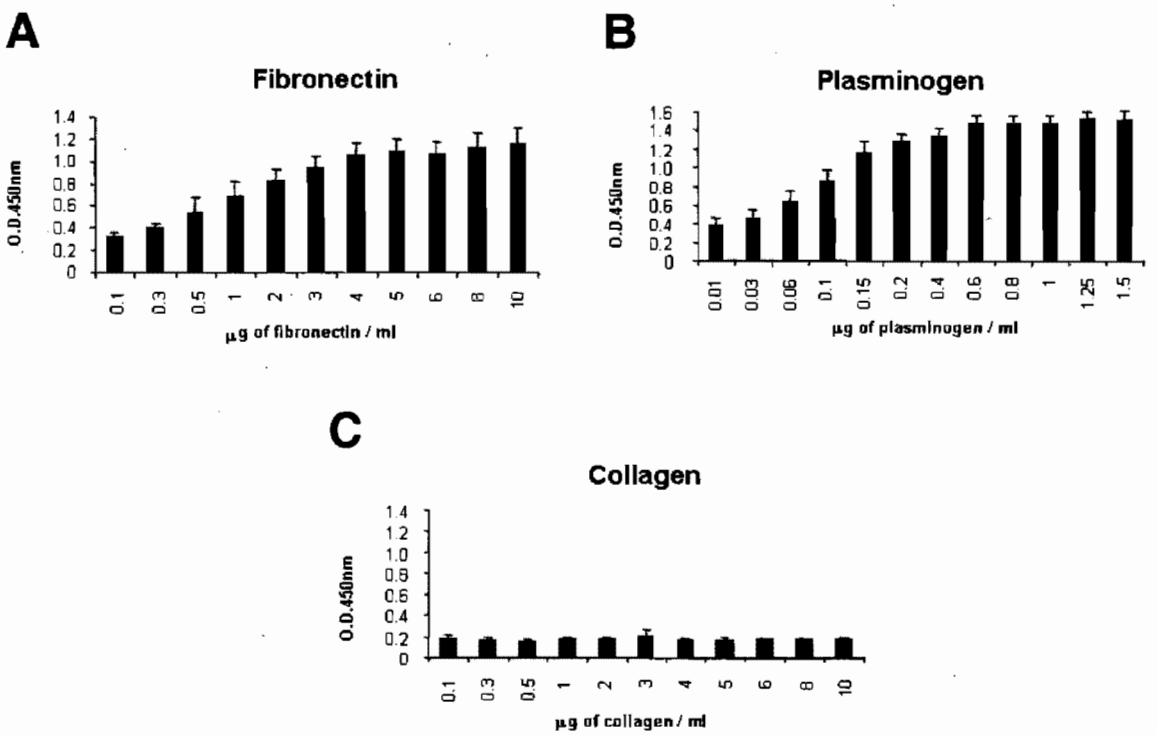

Fig. 1. Representative ELISA analysis for the binding to 5 ug/ml SsEno binding to immobilized collagen. Binding was evaluated after 2 h. Fibronectin and plasminogen was used as positive controls for adhesion. Data are expressed as means ± standard deviations of at least three experiments performed in triplicate.

ANNEX 6

Identification of genes associated with the collagen-binding activity of *Streptococcus suis* using random insertional mutagenesis

As explained before, the ECM proteins have been identified as an important host receptors involved in the bacteria-host adhesion (5). We have identified for the first time in this work that *S. suis* is also able to bind to collagen. Collagen is a polypeptide substance comprising about one third of the total protein in mammalian organisms (3). It is the main constituent of skin, connective tissue, and the organic substance of bones and teeth (3). In fact, different forms of collagen are produced in the body but all consist of three alpha-polypeptide chains arranged in a triple helix (1). The predominant form is Type I collagen. This fibrillar form of collagen represents over 90 percent of our total collagen. Type II collagen is the form that is found exclusively in cartilaginous tissues. It is usually associated with proteoglycans or "ground substance" and therefore functions as a shock absorber in our joints and vertebrae. Type III collagen is also found in our skin as well as in blood vessels and internal organs. In the adult, the skin contains about 80-percent Type I and 20-percent Type III collagen. In newborns, the Type III content is greater than that found in the adult. It is thought that the supple nature of the newborn skin as well as the flexibility of blood vessels is due in part to the presence of Type III collagen. During the initial period of wound healing, there is an increased expression of Type III collagen (2). Type IV collagen is found in basement membranes and functions as a filtration system. Because of the complex interactions between the Type IV collagen and the noncollagenous components of the basement membrane, a meshwork is formed that filters cells as well as molecules and light. Finally, type V collagen is found in essentially all tissues and is associated with Types I and III. In addition, it is often found around the perimeter of many cells and functions as a cytoskeleton.

Although we have described that *S. suis* is able to bind to collagen, up to now any collagen-binding protein has been identified for this pathogen. The main objective of this project was to identify the *S. suis* collagen-binding proteins or the genes involved in this binding process.

To permit its identification, a Tn917 mutant library produced from *S. suis* serotype 2 strain p1/7 was used (4). This library consists in about 1800 mutants each of one containing only one transposon insertion. For the identification of each different mutant, each bacteria composing the bank was named: letter (from A to P), number (1 to 12), letter (A to H).

Each mutant was tested for its ability to bind to collagen compared with their parental strain using a similar ELISA than those that is reported in the article I of this work. Briefly, collagen was coated to ELISA plate wells and after blocking non specific binding to the well with skimmed milk, each mutant was tested for its ability to bind to collagen using an specific polyclonal anti-*S. suis* antibody.

ELISA results of all 1800 mutants were illustrated in Fig. 1A. These results showed that only ten from the initial 1800 mutants have less than 30% of adhesion to collagen type I (red bar) compared with the parental strain P1/7 (Fig 1B). From them, only the E1A mutant has a significant reduction of adhesion to collagen type V (green, Fig 1C). As explained in the introduction, different types of collagen were used because each one represents a different way that can be used by *S. suis* to bind to the host tissues. It is important to note that an ELISA where each one of the ten mutants selected in the first step (Fig. 1A) were coated to the plate and incubated with *S. suis* serotype 2-specific rabbit antiserum was done. This ELISA was used as a control to verify that all mutants react in a similar form with the antibody used in this work and that differences in adhesion are not due to differences in bacterial recognition by this specific antibody. Figure 1B demonstrates that all these ten mutants were recognized by the specific antibody against-*S. suis* in a similar form corroborating that the observed decrease in the adhesion to collagen is directly linked with the transposon insertion in the genome (Fig. 1B).

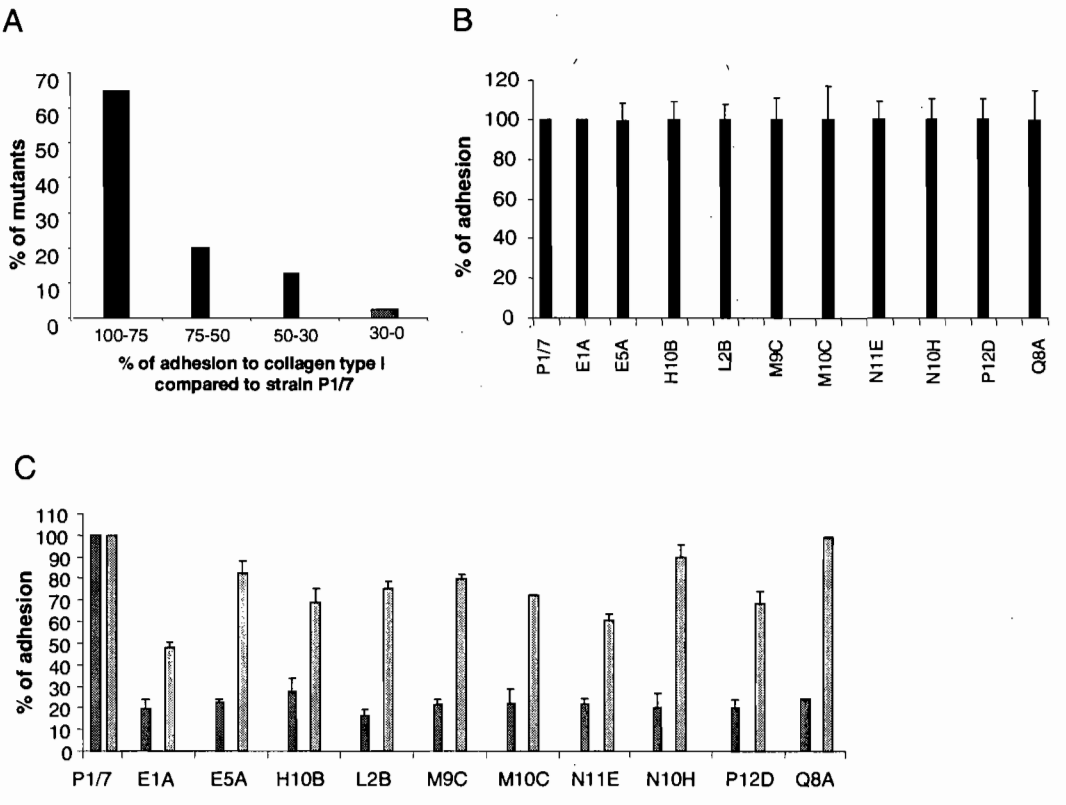

Fig. 1. Adhesion of Tn917 mutants to collagen. Only 10 mutants have less than 30% of adhesion to collagen compared with strain P1/7 (A). All these 10 mutants react with specific anti-S. suis polyclonal antibody in a similar way (B). ELISA results of the adhesion of the 10 mutants with collagen type I (in red) and to collagen type III (in green)

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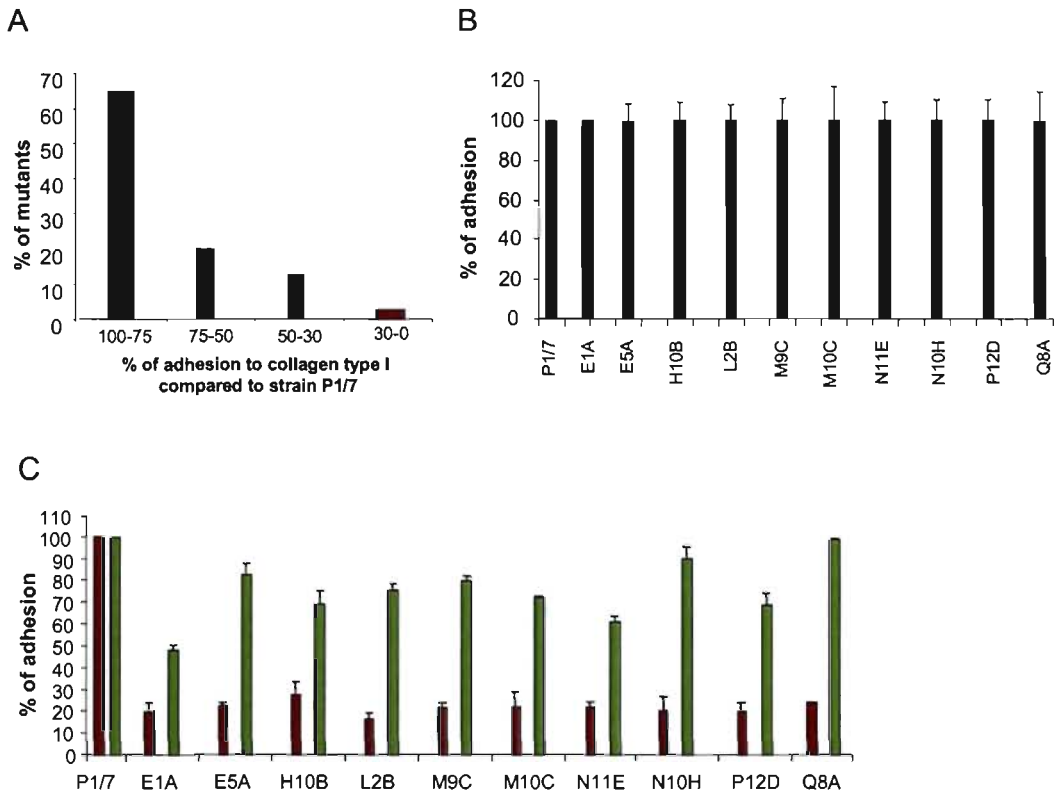

Fig. 1. Adhesion of Tn917 mutants to collagen. Only 10 mutants have less than 30% of adhesion to collagen compared with strain P1/7 (A). All these 10 mutants react with specific anti-S. suis polyclonal antibody in a similar way (B). ELISA results of the adhesion of the 10 mutants with collagen type I (in red) and to collagen type III (in green)

Once isolated from the rest of mutants, the Tn917 mutated genes from the 10 strains with decreased adhesion to collagen were identified by Plasmid rescue (Fig 2).

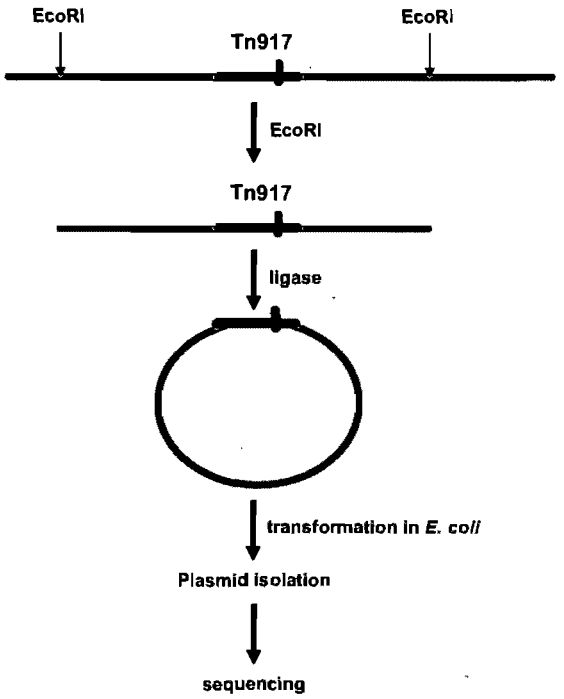

Fig. 2. Diagram of plasmid rescue technique. The genes targeted and mutated with Tn917 were identified by digestion of genomic DNA of each strain with EcoRI restriction enzyme, ligation of fragments with T4 ligase, transformation to *E. coli*, isolation of Tn917 containing plasmids by resistence to ampicillin and finally, sequencing of the gene using specific primers of the Tn917.

Plasmid rescue is a technique in which transposon disturbed genes can be identified by digesting the bacterial genome with a specific restriction enzyme (in our case EcoRI), relegation of the fragments obtained and transformation of the obtained "plasmids" in a host bacteria such as *E. coli*. As transposon contains a resistance gene for an antibiotic such as ampicillin, only bacteria containing DNA fragments with the transposon can grow in the presence of antibiotic. Once bacteria are isolated by this technique, the genes can be sequenced using specific primers localized in the transposon. Later, biocomputacional analysis using a Blast server can be used to identify the genes corresponding to the sequence obtained after sequentiation.

Our results revealed that different genes could be implied in the adhesion of *S. suis* to collagen. The genes identified are listed in Table 2. From all the genes identified, none of them has a typical bacterial collagen-binding protein sequence. Moreover, non of them can be identified as a known gene involved in the export of adhesins to the surface. Regarding the literature any relationship between these genes and collagen has been found, so further research is necessary to

find the concrete role of these genes in the adhesion of *S. suis* to collagen. In addition, in 2 of the ten mutants was not possible to identify the gene by this method and other methods have to be done to determine which gen had been mutated by the transposon.

NOM	Cn I	Cn V	Fn	identification
E1A	14 %	50 %	26 %	plasmid
E5A	25 %	85%	77 %	Excinuclease ABC
H10B	30 %	75 %	68 %	ORF2 (Aro operon)
L2B	12 %	80 %	80 %	plasmid
M9C	20 %	83%	97 %	Auxin efflux protein
M10C	12 %	85 %	96 %	Copper-translocating P-type ATPase
N11E	15 %	82 %	93 %	α/β hydrolase fold
N10H	13 %	93 %	89 %	Surface protein, anchor region *S. suis* FlpS gene (arginine Deim operon)
P12D	15 %	79 %	86 %	Bacteriocin operon component ScnE
Q8A	17 %	100 %	100 %	Transglutaminase-like

Table. 2. List of genes identified by plasmid rescue and Blast.

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ANNEX 7: ARTICLE IV

Immunization with recombinant Sao protein confers protection against *Streptococcus suis* infection

Yuanyi Li[1], Marcelo Gottschalk[1], Miriam Esgleas[1], Sonia Lacouture[1], J. Daniel Dubreuil[1], Philip Willson[2], and Josee Harel[1*]

[1]Groupe de Recherche sur les Maladies Infectieuses du Porc, Faculté de médecine vétérinaire, Université de Montréal, C.P. 5000, St-Hyacinthe, Québec J2S 7C6, Canada.
[2]Vaccine and Infectious Disease Organization, University of Saskatchewan, 120 Veterinary Road, Saskatoon, Saskatchewan S7N 5E3, Canada

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Role of the candidate in the conception of this article:

I have actively participated in conception, the design of the experiments, the laboratory work, the analysis of results and the figure conception concerning the opsonophagocytosis of *S. suis* showed in this article.

ABSTRACT

Sao is a *Streptococcus suis* surface protein recently identified as a potential vaccine candidate. In this study, recombinant Sao in combination with Quil A provided cross-protection against *S. suis* serotype 2 disease in mouse and pig vaccination protocols. Subcutaneous immunization of mice elicited strong immunoglobulin (Ig) G antibody responses. All four IgG subclasses were induced, with the IgG2a titer being the highest followed by IgG1, IgG2b and IgG3. Challenge of the mice with *S. suis* strain 31533 resulted in a mortality of eighty percent in the control group which received Quil A only. In contrast, all the mice immunized with Sao survived. In a pig vaccination protocol, intramuscular immunization with Sao also elicited significant humoral antibody response, and both IgG1 and IgG2 subclasses were induced with a predominance of IgG2 production. *In vitro* assay showed that Sao-induced antibodies significantly promoted the ability of porcine neutrophils in opsonophagocytic killing of *S. suis*. An aerosol challenge of the pigs with *S. suis* strain 166 resulted in clinical signs characteristics of *S. suis* infection in diseased pigs. The vaccine group showed significantly better survival, lower clinical score and less *S. suis* recovery from postmorterm tissue samples compared with the control group. Furthermore, this study also revealed that although challenge *S. suis* strains express Sao size variants, recombinant Sao conferred cross-protection. These data demonstrate that recombinant Sao formulating with Quil A triggers strong opsonizing antibody responses which confers an efficient immunity against challenge infection with heterologous *S. suis* type 2.

INTRODUCTION

Streptococcus suis is an important pathogen of swine causing meningitis, septicaemia, arthritis, endocarditis, pneumonia and substantial economical losses in the swine industry worldwide (17, 23, 47). It is also an important zoonotic agent for humans in contact with diseased pigs or their products, causing life-threatening diseases as reported in a recent outbreak in China (43). Thirty-five serotypes have been described so far (17). Serotype 2 is the most prevalent type in association with diseases in most countries. The pathogenesis and virulence attributes of *S. suis* are not well defined and attempts to control the infection are hampered by the lack of an effective vaccine (22).

Different types of vaccines have been developed or are presently under investigation. At present, inactivated commercial autogenous vaccines are used in the field, but results have been inconsistent (20, 35). Furthermore, safety data for autogenous vaccines are lacking, which has liability implications for the use of this type of material (19). Attenuated or avirulent live *S. suis* strains have been tested and results were also equivocal (6, 26, 53). In addition to bacterins and live vaccines, a number of purified bacterial components have been developed as vaccine candidates. The capsule polysaccharide is a critical virulence factor of *S. suis*. However, a vaccine based on capsular material was unsatisfactory due to its poor immunogenicity (13). A vaccination strategy using purified suilysin (27), or muramidase-released protein (MRP) and extracellular protein factor (EF) (54) from *S. suis* serotype 2 have been shown to protect pigs from homologous and heterologous serotype 2 strains. However, a substantial number of virulent strains in some geographic regions do not express these proteins (14, 16, 45).

We recently identified a surface protein (Sao) which is highly conserved among *S. suis* species (37). Convalescent swine sera have high titers of antibody against this protein, suggesting that Sao is a potent immunogen that is expressed during *S. suis* infection. These findings made the Sao a candidate for use in a subunit vaccine. However, in a convenient test, immunization of piglets with recombinant Sao mixed with the oil-in-water Emulsigen® triggered a predominant production of IgG1, and these antibodies lacked opsonophagocytic function and did not confer protection (37). This suggested that the quality of the type 1/type 2 immune response bias was inappropriate to mediate protection against *S. suis*. It is known that host protection against infection caused by *S. suis*, a highly encapsulated microorganism, is mediated primarily by opsonophagocytosis which is mainly associated with a Th1-type immune response characterized by IgG2a production (5, 17). Vaccine formulation and components, such as adjuvants, can dramatically influence a vaccine-

induced antibody response including bias to type 1 or type 2 responses which may have a significant effect on the protective efficacy of a vaccine (1, 31, 44). Evidence from vaccination using surface antigens of other Gram-positive bacteria indicated that the efficiency of antibody-mediated opsonophagocytosis and protection can be dramatically improved by using Th1-directing adjuvants to promote a Th1-type immune response (2, 36). We therefore hypothesized that Sao may be protective in a vaccination protocol involving optimal adjuvant and higher antigen dosage. In this study, the efficacy of recombinant Sao in combination with Quil A was demonstrated by protection against *S. suis* infection and disease in mice, as well as in pigs, the target species of this vaccine candidate.

MATERIAL AND METHODS

Bacterial strains

Three *S. suis* strains of serotype 2 were used in this study. Strain S735 was used to clone the *sao* gene and to produce the protein (37). This strain is the reference strain and was originally isolated from a diseased pig in the Netherlands (28). Strains 166 and 31533 originated from pigs with meningitis (4, 33). Strain 31533 was chosen for challenging mice and strain 166 was used for challenging pigs. Bacteria were grown on plates made of Todd-Hewitt broth (Difco, Detroit, MI, USA) containing 2% agar or in liquid cultures of Todd-Hewitt broth.

Immunization and challenge of mice

Recombinant Sao was produced and purified as previously described (35). Six-week-old female CD-1 mice were randomly assigned (according to their body weight) to two groups of ten, and immunized subcutaneously twice at a 2-week interval with either 20 μg of purified Sao mixed with 20 μg of Quil-A (Brenntag Biosector, DK-3600 Frederikssund, Denmark) adjuvant or 20 μg of Quil-A only as a control in 100 μl of phosphate-buffered saline (PBS) per mouse. Ten days after the second vaccination, the animals were challenged intraperitoneally with 1 x 10^8 CFU per mouse of log-phase *S. suis*, strain 31533, in 1 ml of Todd-Hewitt broth. This challenge model was confirmed to reproduce septic shock and meningitis similar to those induced by *S. suis* in pigs (unpublished data). Mice were monitored daily for clinical signs such as abnormal behaviour, rough hair coat, ataxia and mortality, until day 14 after the infection. Blood samples were collected prior to each vaccination and the challenge for determining antibody responses. Guidelines from the "guide to the care and use of experimental animals" of the Canadian Council on Animal Care were followed during the experiment, which followed a protocol that had been approved by the University of Montreal committee on animal care.

Immunization and challenge of pigs

Pigs were used to perform the immunization and protection experiment at the Vaccine and Infectious Disease Organization (VIDO, University of Saskatchewan, Saskatoon, Canada) in accordance with principles outlined in the "guide to the care and use of experimental animals" of the Canadian Council on Animal Care using a protocol that was approved by the University Committee on Animal Care. Four week-old piglets with average weight of 7.79 kg from a herd that is free of *S. suis* serotype 2 were randomly assigned to two groups of twelve. Animals were injected intramuscularly twice at a 2-week interval with 1 ml of either 200 μg purified Sao mixed with 400 μg Quil A or 400 μg Quil A only in physiological saline as a control. Two weeks after the second

injection, the immunized and control animals were challenged by aerosol of 1 ml (6.8×10^6 CFU) of a log-phase culture of *S. suis* strain 166 as previously described (39). Blood samples were collected prior to each injection and challenge for determination of antibody responses. Pigs were monitored for body temperature, clinical signs and mortality for ten consecutive days after challenge. A daily clinical score (from 0 to 8) was derived as the sum of attitude and locomotion scores for each pig based upon signs of nervous, musculoskeletal or respiratory disease as follows. Attitude: 0 = normal attitude and response to stimuli; 1 = inactive and slow to respond, and oculo-nasal secretions; 2 = only responsive to repeated stimuli; 3 = recumbent, nonresponsive, unaware of surroundings; 4 = dead. Locomotion: 0 = normal gait and posture; 1 = slight incoordination, lameness and/or joint swelling but rises without assistance; 2 = clearly uncoordinated or lame but stands without assistance; 3 = severe lameness and/or severe ataxia; 4 = dead. Pigs having a clinical score greater than 2 on either scale were euthanized by lethal injection. Postmortem examination procedure was conducted for all pigs. Brain, tracheobronchial lymph node and grossly affected joint samples from all pigs and blood samples from euthanized pigs were cultured for bacteria recovery.

Enzyme-linked immunosorbent assay (ELISA)

Titers of Sao-specific total IgG and IgG subclasses in mouse and swine sera were determined by ELISA as previously described (37). Briefly, Polysorb plates (Nunc-Immunoplates, Rochester, NY, USA) were coated overnight at 4°C with 100 µl per well of the purified recombinant Sao at a concentration of 0.3 µg/ml in carbonate buffer. The plates were incubated with serial dilutions of test sera in PBS containing 0.05% Tween-20 (PBS-T) for 1.5 h at room temperature. For determination of antibodies in mice, bound antibodies were detected by incubation with peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3 antisera (Serotec, Kidlington, Oxford, UK) for 1 h at room temperature. For determination of swine total IgG, bound antibodies were detected by incubation with peroxidase-conjugated goat anti-swine IgG(H+L) antisera (Jackson Immuno Research Laboratories, Inc., West Grove, PA) for 1 h at room temperature. For swine IgG1 and IgG2 detection, mouse anti-porcine IgG1 or IgG2 (Serotec) were used as the primary antibodies, and peroxidase-conjugated goat anti-mouse IgG(H+L) (Serotec) was used as the secondary antibody. The plates were developed with TMB substrate (Zymed, S. San Francisco, CA, USA). Absorbance was measured at 450 nm in an ELISA reader (Power Wave 340, Bio-Tek Instruments, Inc., Winooski, VT, USA). The serum dilution that resulted in an OD_{450} reading of 0.1 after background subtraction was considered the titer of this serum.

Opsonophagocytosis assay

To investigate the role of Sao-specific antibody in protection, total IgG was purified using a protein-A column (Pharmacia, Uppsala, Sweden) from sera pools of control or Sao-vaccinated pigs after the second immunization. Porcine neutrophils were isolated from pigs that belonged to a high health status herd. Complete normal serum from the healthy pig was used as a source of complement. An opsonophagocytosis assay was then performed as previously described (Chabot-Roy, 2006). Briefly, *S. suis* 166 strain was suspended in complete normal porcine serum containing 25 μg/ml of the purified IgG from either Sao-vaccinated pigs or control pigs, and pre-opsonized for 30 min at 37 °C. Neutrophils at a concentration of 5×10^6 cells/ml were mixed with 1×10^4 CFU/ml of the bacterial in microtubes and incubated 90 min at 37 °C with 5% CO_2. The neutrophil cells were lysed with sterile water and viable bacterial counts were performed on THA plates. Tubes with bacteria alone were treated similarly and used as controls. The tests were performed eight times. Results are expressed as percentage of killed bacteria.

Western blot

Fifty microliters of *S. suis* culture supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 8% acrylamide gel. Proteins transferred to nitrocellulose membrane (Bio-Rad, Mississauga, Ontario, Canada) were detected by incubating overnight at 4 C° with 1/200 dilution of the pooled sera from the mice which received two doses of Sao or from the control animals. in TBS-0.05% Tween- 20 containing 5% skim milk. Sao-specific antibody was detected with peroxidase-conjugated goat anti-mouse IgG (Serotec), and visualized using 4-chloro-1-naphthol (Sigma, St. Louis, MI, USA) as the substrate.

Polymerase chain reaction (PCR) and sequences

S. suis cell pellet from 5 ml overnight culture was suspended in 1 ml of lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 3% SDS, 1 mg/ml RNase, pH 8.5) and transferred to a 2 ml screw cap tube containing 0.5 g of 0.1-mm glass beads (BioSpec, Oklahoma, USA). The suspension was homogenized for 3 min at maximum speed using the Mini-Bead Beater (BioSpec). The sample was centrifuged for 3 min at 16000 x g in a microcentrifuge, and then the supernatant was used to extract the genomic DNA following standard procedure. The complete *sao* gene was amplified from the genomic DNA using pS1F (5'-ATGAATACTAAGAAATGG-3') and pS1R (5'-AATTTACGTTTACGTGTA-3') primer pair, and the DNA fragment flanking the repeating region in *sao* was amplified using pS2F (5'-GAAATATCGAACCCCCTAAAG-3') and pS2R (5'-CTTCGACTGTACCATTTTGGT-3') primer pair. The PCR was performed for 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 30 s at 46°C, and 1 min at 72 °C in a thermal cycler

(Eppendorf Scientific Inc, Hamburg, Germany). The amplicons were analyzed on 0.8% agarose gel and sequenced using the same primers.

Statistics

Comparison between antibody titers and percentage of killed bacteria was done using *t* test. The clinical scores were transferred by ranking, and the significance of the difference between groups was determined by *t* test. Survival distributions were evaluated with chi-square analysis using the Kaplan-Meier method and the significance of the difference was tested using the Log-rank test. Fisher Exact Test was applied to compare the proportion of postmortem tissues from which *S. suis* was recovered. A P value of < 0.05 was taken as significant.

RESULTS

Sao-specific IgG and IgG subclasses

Subcutaneous immunization of mice with Sao in combination with Quil A elicited a significant humoral IgG response after primary immunization. The second immunization boosted the specific antibody response which was significantly higher than the level after the primary immunization ($P<0.001$) (Fig. 1A). Furthermore, all four tested IgG subclasses were induced in Sao-immunized mice, with the IgG2a titer being the highest followed by IgG1, IgG2b and IgG3 as measured in the sera nine days after the second vaccination. The IgG2a titer was significantly higher than the titers of other IgG subclasses, including IgG1 ($P<0.05$) (Fig. 1B). In contrast, the Sao-specific IgG and its subclasses in sera of the mice before vaccination and the mice in control group were below the limit of detection.

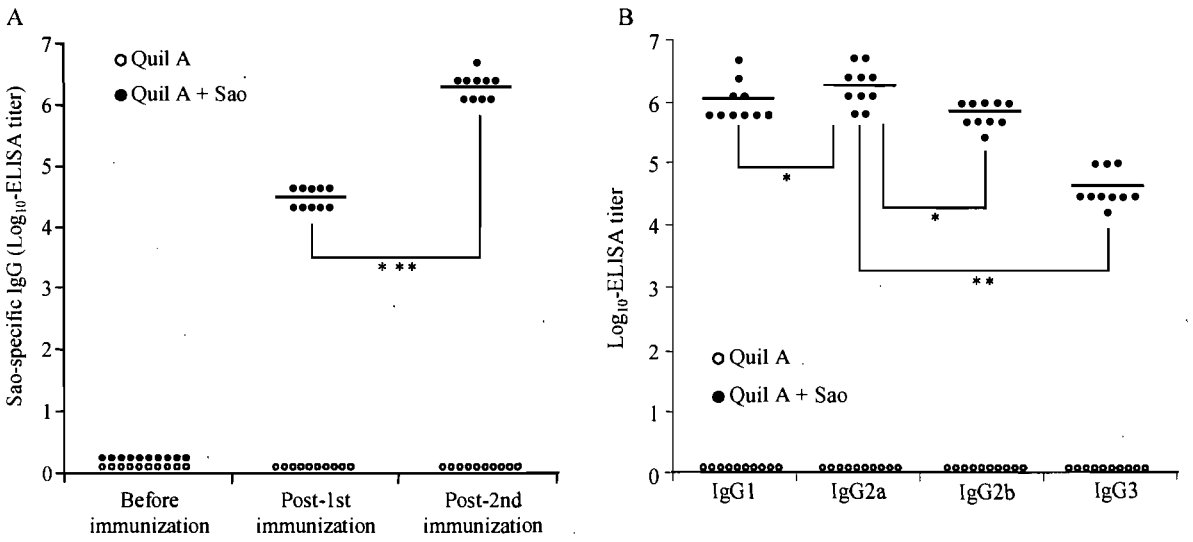

Fig. 1. Serum antibody responses in mice immunized with Quil A (open cycles) or Quil A plus recombinant Sao (solid cycles). (A) Total Sao-specific serum IgG. (B) IgG subclasses in sera nine days after the second immunization. Antibody titers for individual mice are shown, with the average titer (n=10) represented as a bar. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Similar patterns of immune responses were revealed in pigs that intramuscularly received 200 μg of Sao in combination of 400 μg of Quil A. Primary immunization of pigs triggered a strong antigen-specific IgG response. The serum IgG titer was significantly higher than that in control pigs received only Quil A and the pigs before immunization ($P<0.01$). After a second dose of the vaccine, a significant increase in IgG level ($P<0.001$) was seen in Sao-immunized pigs (Fig. 2A).

Assessment of IgG subclasses demonstrated that while both IgG1 and IgG2 subclasses were induced in sera of Sao-immunized animals, the IgG2 response significantly dominated the IgG1 response ($P<0.05$) (Fig. 2B).

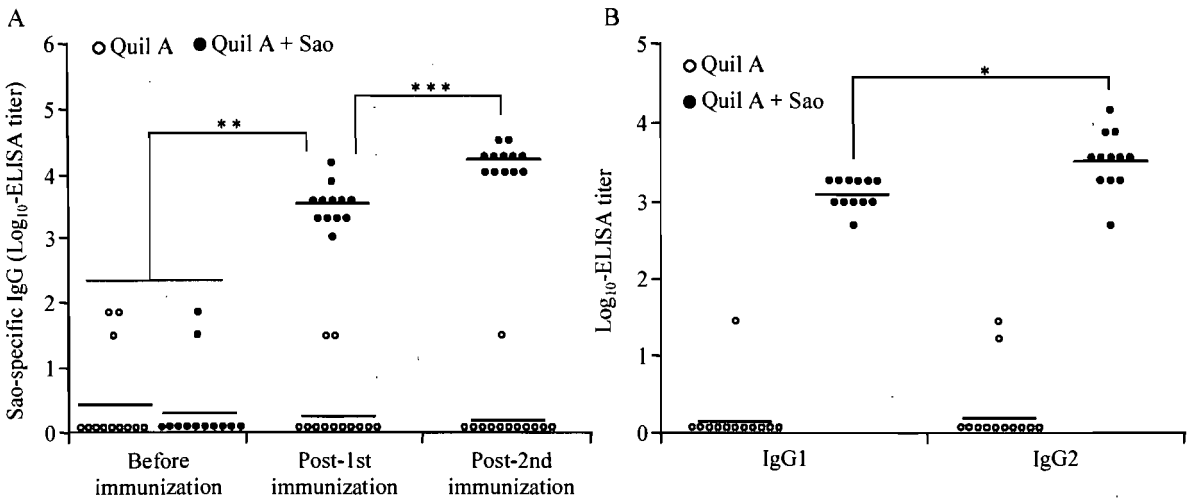

Fig. 2. Serum antibody responses in pigs immunized with Quil A (open cycles) or Quil A plus recombinant Sao (solid cycles). (A) Total Sao-specific serum IgG. (B) IgG subclasses in sera thirteen days after the second immunization. Antibody titers for individual pigs are shown, with the average titer (n=12) represented as a bar. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Protection of mice against *S. suis* strain 31533

Sixteen hours after administering the challenge infection with *S. suis* 31533, all mice in the non immunized control group exhibited clinical signs, such as ruffled hair coat suggesting fever and slow response to stimuli. Beginning about four days after the challenge, eight of ten mice in this group successively developed severe central nervous system signs such as running in circles and opisthotonos. All eight of the ill mice died, or met criteria for humane euthanasia due to the severity of their condition. In contrast, most of the mice in Sao-vaccinated group showed only mild and transient rough hair after the challenge, and all mice survived the *S. suis* infection, resulting in complete protection from cause-specific mortality ($P<0.001$) (Fig. 3).

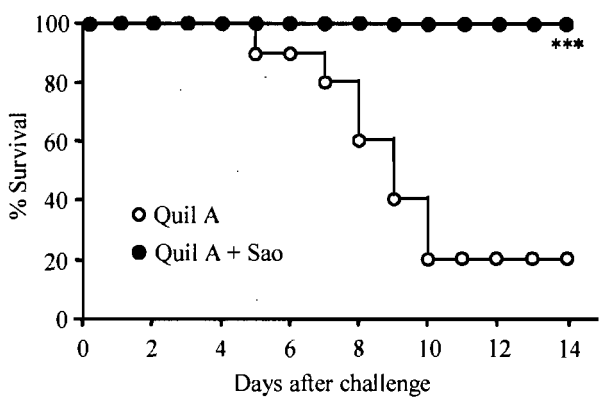

Fig. 3. Survival of mice immunized with Quil A (open circles) or Quil A plus recombinant Sao (solid circles) following challenge with *S. suis* 31533. Each group consists of ten mice. ***, $P<0.001$.

Protection of pigs against *S. suis* strain 166

One pig in Sao-immunized group was humanely killed because of an unrelated disease and this pig was excluded from analysis of the effects of vaccination on disease. Aerosol challenge of the pigs with *S. suis* 166 strain resulted in diseases characteristic of *S. suis* infection. The mean accumulated clinical scores of two groups are presented in Fig. 4A, and clinical signs were significantly less severe in the Sao-vaccinated group compared with the control group ($P<0.05$). The body temperature data showed no significant difference between the two groups, although the Sao-vaccinated group tended to have lower temperatures (data not shown). In the control group, three pigs died and four more were euthanized due to high clinical scores prior to the end of the experiment, resulting in a survival rate of 42%. In contrast, only two pigs in Sao-vaccinated group were euthanized with a survival rate of 82%. Comparison of survival curves showed that survival time in the Sao-vaccinated group was significantly longer than that in the control group ($P<0.05$) (Fig. 4B). Bacterial culture of samples from blood, brain, tracheobronchial lymph node and joint was done to monitor the infection level and the recovery of bacteria with colonial morphology typical of the challenge strain was summarized in Table 1. Although the number of organs with detectable bacteria in the Sao-vaccinated group was less than in the control group, only the proportion of positive brain tissue samples from immunized pigs was significantly lower than from control pigs ($P<0.01$) (Table 1).

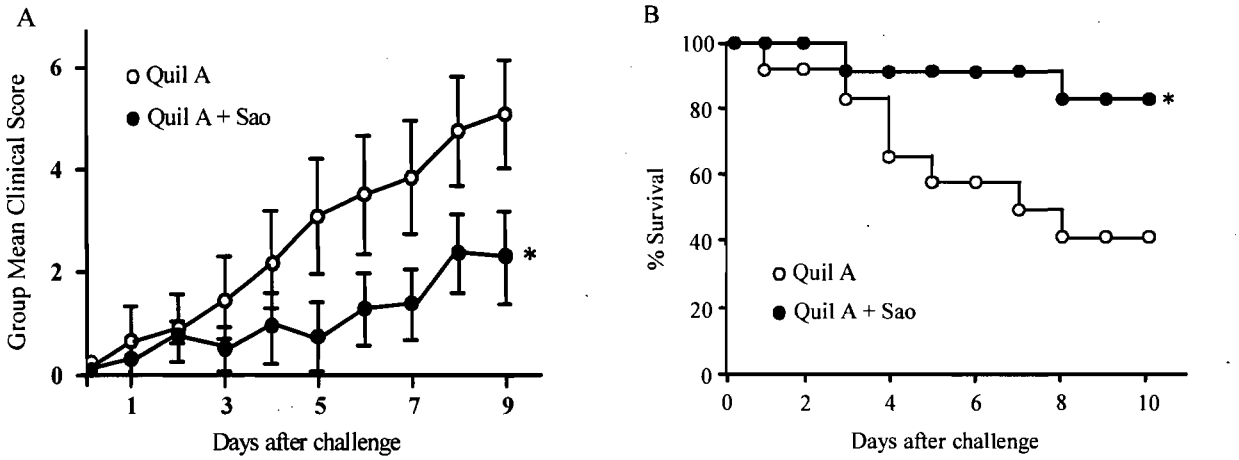

Fig. 4. Protection of pigs immunized with Quil A (open circles) or Quil A plus recombinant Sao (solid circles) following challenge with *S. suis* 166. (A) Clinical scores (daily mean and standard deviation) of pigs after challenge. (B) Survival of pigs after challenge. The data are reported for eleven pigs in the Sao-vaccinated group and twelve pigs in the control group, respectively. *, $P<0.05$.

Sample	No. of *S. suis* recovery/total no. of samples examined		*P* value
	Quil A	Quil A + Sao	
Blood	4/9[a]	1/11	0.13
Brain	10/12	2/11	0.003 **
Lymph node	8/12	6/11	0.68
Joint	4/12	2/11	0.64

Table 1. Bacteriological analysis of postmortem samples from pigs immunized with Quil A or Quil A plus recombinant Sao. [a] Postmortem blood samples could not be obtained from three pigs that died before they were observed. **, $P<0.01$.

Functional activity of Sao-induced antibodies

To determine the nature of protection, serum antibodies obtained from Quil A plus Sao-vaccinated pigs were compared with antibodies from pigs that received Quil A only for their ability to promote opsonization phagocytosis and killing of *S. suis* 166 by porcine neutrophils in vitro. As shown in figure 5, antibodies from Sao-vaccinated pigs mediated significantly more efficient opsonophagocytic killing of *S.suis* than antibody from pigs that received adjuvant alone ($P<0.001$).

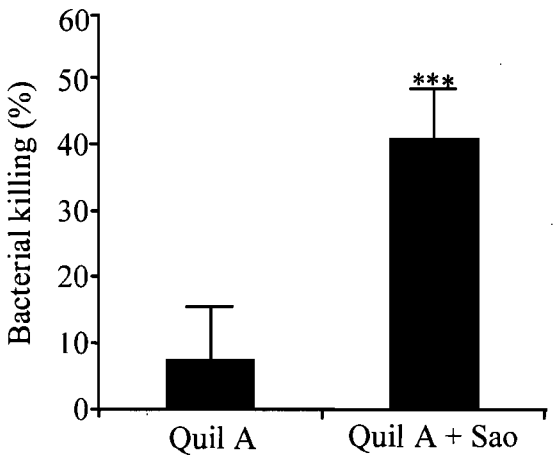

Fig. 5. Effect of antibodies on opsonophagocytic killing of *S.suis* by porcine neutrophils. The antibodies were purified from pooled sera of pigs immunized with Quil A or Quil A plus Sao. Data are expressed as mean percentage ± standard deviation of killed bacteria and are representative of eight independent experiments. *, $P<0.001$.

Immune recognition of Sao protein and its variants

Specificity of Sao-induced antibodies was demonstrated by western blot with sera pooled from Sao-immunized mice, which recognized the Sao protein of wild type *S. suis* strains as well as the recombinant Sao (Fig. 6). In contrast, sera from non immunized control mice did not react with the proteins (data not shown). However, size variation of Sao was noted among the *S. suis* strains. While *S. suis* S735 strain, from which the Sao protein antigen was produced, expressed an apparent 110 kDa Sao protein similar in size to the antigen, strain 166 and 31533 expressed Sao variants of approximately 100 kDa and 93 kDa, respectively (Fig. 6).

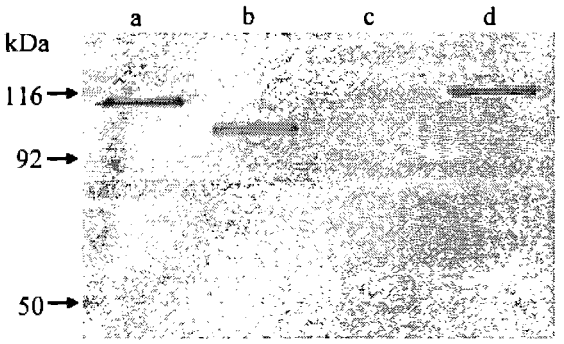

Fig. 6. Western blot showing the variation in Sao protein of *S. suis*. Culture supernatants of *S. suis* strain S735 (lane a), strain 166 (lane b), strain 31533 (lane c), and the purified recombinant Sao that was used for immunization (lane d) were separated by SDS-PAGE and then transferred to membrane. Blot was detected with sera pooled from mice after the second immunization with recombinant Sao. The molecular masses are indicated on the left.

Genetic analysis of Sao variants

The genes encoding Sao variants were analyzed by PCR. As shown in Fig. 6, the size of *sao* varied among *S. suis* strains S735, 166 and 31533, and corresponded to the size of the Sao variants observed in the western blot. One of the features of Sao protein is the presence of ten repeats of a 27-amino-acid sequence separated by 3-amino-acid spacers near the C-terminal (37). Analysis of the PCR fragments spanning the repeating region of *sao* suggested that a variable number of the repeats accounted for the size differences of *sao* (Fig. 7). Indeed, DNA sequence analysis revealed a deletion of 270 bp nucleotide sequence corresponding to three repeats plus the spacers in strain 166 and a deletion of 450 bp sequence corresponding to five repeats plus the spacers in strain 31533, compared with the sequence of *sao* in strain S735.

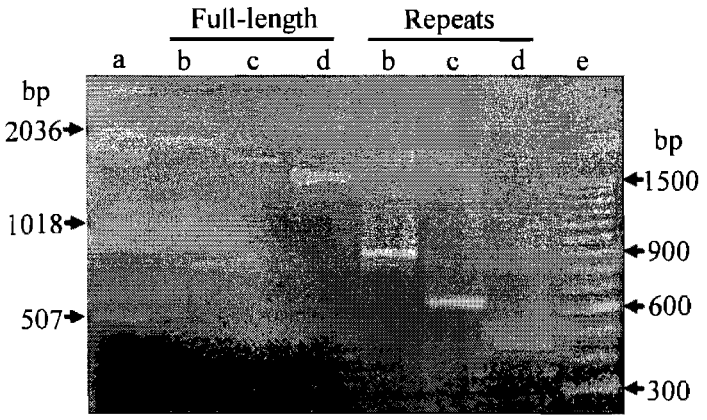

Fig. 7. PCR amplification products of the full-length of *sao* gene of *S. suis* and the DNA fragments flanking the repeats of *sao*. The variations of complete *sao* were correspondingly revealed in the differences of the DNA fragments spanning the repeats. Lanes: (a) 1 kb DNA ladder (Life Technologies), (b) strain S735, (c) strain 166, (d) strain 31533 and (e) 100 bp DNA ladder (Invitrogen).

DISCUSSION

In our continued effort to search for *S. suis* protein(s) useful in the development of a vaccine, a highly immunogenic surface protein, Sao, was identified from a virulent strain (S735) of *S. suis* serotype 2. In a convenient pig immunization protocol, Sao in combination with Emulsigen triggered a predominant production of IgG1 that did not confer the protection against *S. suis* challenge infection (37). In this study, we first used a mouse model to test the protective efficacy of recombinant Sao in combination with Quil A and found that the experimental vaccine induced a predominant generation of IgG2a which confers protection against *S. suis* infection. This prompted us to further evaluate the protective capacity of Sao in combination of Quil A in pigs, the target host of this potential vaccine. Indeed, it significantly protected the pigs against *S. suis* infection and disease.

Induction of the appropriate type of antigen-specific immune responses is crucial for the success of vaccines. The IgG subclass produced as a consequence of immunization reflects the type of immune responses. In mice, serum IgG1 is associated with Th2-type response, whereas serum IgG2a is associated with a Th1-type response which is particularly effective at mediating bacterial opsonophagocytosis (49). Of the mouse IgG subclasses, IgG2a is the most effective at binding to the FcγRI on phagocytic cell (42, 49). Thus, it is likely that predominant IgG2a production in this current mouse vaccination protocol contributed most to the observed protection. However, it is also possible that IgG2a is not the only effector of protection induced by vaccination with Sao. Some studies have shown that IgG2b and IgG3 are also associated with Th1-type immune response and are critically involved in bacterial opsonophagocytosis and protection against infection of Gram-positive pathogen (9, 32, 41, 48). In this pig immunization and challenge protocol, the Sao-induced immune response was characterized by predominant IgG2 production. Although the concept of "Th1/Th2" balance is not yet well documented in pigs, recent evidence showed that porcine IgG2 had greater complement activating ability than did IgG1 (11).

Adjuvants play an important role in the efficacy of vaccines. The type of adjuvant used can direct the type of immune response generated to an administered antigen (44). It has been previously shown that an appropriate adjuvant is essential in determining the outcome of vaccination, and that protection following vaccination was obtained only after switching immune responses to a predominantly Th1 type, such as the vaccines against *Streptococcus pneumoniae* (2, 40), *Mycobacterium tuberculosis* (38), *Chlamydia pneumoniae* (3) and *Brucella abortus* (21). The adjuvant Quil A has been shown to enhance the antibody levels, and more importantly to shift

response towards type 1, thus resulting in the induction of both bactericidal and opsonophagocytic antibodies (12, 30, 31, 51). In this modified pig protocol, it appears that Sao combined with Quil A triggered an adequate immune response bias which consequently led to protection. To determine the nature of protection, Sao-induced antibodies were analyzed for their ability to promote opsonophagocytic killing of *S. suis* in the presence of white blood cells, an important immunological correlate of protective immunity against *S. suis* (7). We found that antibodies purified from the sera of pigs that received two doses of Sao vaccine in combination of Quil A exhibited strong opsonic capacity. Given our previous study in that Sao combined with Emulsigen® triggered a predominant production of IgG1 and these antibodies lacked opsonophagocytic function (37), this result indicated that Sao in the present formulation may more adequately induce protective antibodies that are capable of triggering leukocyte effector. The enhanced level of opsonizing antibodies is likely related to the predominant generation of IgG2, detected by using Quil A adjuvant. However, it should be emphasized that the type of immune response induced could be also affected by the antigen dose (10, 15, 52). Comparing with our previous study in which 100 μg/per pig of Sao was used, a dosage of 200 μg was applied in this trial. Although the exact factor(s) crucial in directing the immune response toward the adequate bias was not defined, this study did provide a basis of suitable formulation for further clinical evaluation of Sao protein as a vaccine candidate for control of *S. suis* disease in pigs.

S. suis strain S735, from which the *sao* gene was originally cloned, was not used for challenging the animals due to the controversial report about its virulence in experimental infection models (8, 50). We have previously confirmed that a Sao-specific antibody raised in rabbit cross-reacted with cell lysates of *S. suis* strain 31533 (unpublished data) and strain 166 (37). Thus, they were chosen for challenging animals to investigate the cross-protection of recombinant Sao against heterologous *S. suis* field strains. The sera from the animals immunized with recombinant Sao recognized size variants of Sao expressed by *S. suis* field strains, suggesting that differences in Sao among the *S. suis* strains used in this study do

not alter the immune recognition of the recombinant Sao-elicited antibody. One of the features of Sao protein is the presence of a region of ten repeats near the C-terminal (37). Variation of repeat numbers has been commonly observed in bacterial proteins, such as EF of *S. suis* (46), the M-protein of *Streptococcus pyogenes* (18, 24, 25, 29), and alpha-like protein of group B Streptococcus (34). Therefore, we assumed that the size difference of Sao occurred due to variation in the number of repeats. This was confirmed by DNA sequencing. It has been proposed that the size variation of Gram-positive bacterial proteins, such as M-protein, is a mechanism by which organisms can escape from the host immune system (18, 24, 25, 29). However, our study shows that Sao-specific antibody cross-reacted with Sao variants and moreover, Sao vaccine offers cross protection against

S. suis expressing Sao variants. This discrepancy may result from the structural difference between Sao and M-protein. In M-protein, the highly variable repeat region presents in N-terminal halve and the highly conserved region in C-terminal halve (29). As a C-terminal anchored protein, the N-terminus extends outwards from the cell and epitopes close to the C-terminus may be masked by other cell wall components. As a result, variation in the N-terminus alters the ability of certain antibodies, originally produced in response to the parent protein, to bind to the mutant molecules or opsonize the mutant organisms (29). Different from the M-protein, the variable repeat region in Sao is located in C-terminal halve and the conserved region in N-terminal halve (37). Therefore, deletion of some repeats does not render them inaccessible to antibody binding.

In summary, we have shown that recombinant Sao in a vaccine formulation with Quil A triggers strong opsonizing antibody responses which confer protection against experimental *S. suis* infection. In addition, Sao protects from challenging strains expressing Sao size variants. These findings suggest that Sao is a potential candidate for development of a subunit vaccine against *S. suis* infection. However, an optimum vaccine formulation remains to be further studied.

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ANNEX 8: ARTICLE V

Disruption of *srtA* gene in *Streptococcus suis* results in decreased interactions with endothelial cells and extracellular matrix proteins

G. Vanier[a], T. Sekizaki[b,c], M.C. Domínguez-Punaro[a], M. Esgleas[a], M. Osaki[b], D. Takamatsu[b], M. Segura[d], and M. Gottschalk[a],*

[a]Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP) and Centre de Recherche en Infectiologie Porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, 3200 rue Sicotte, Saint-Hyacinthe, Qc, J2S 2M2, Canada. [b]Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan. [c]United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagito, Gifu, Gifu 501-1193, Japan. [d]Centre for the Study of Host Resistance, McGill University Health Centre Research Institute, 1650 Cedar Avenue, Montreal, Qc, H3G 1A4, Canada

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Role of the candidate in the conception of this article:

I have actively participated in conception, the design of the experiments and the laboratory work concerning the adhesion of SrtA mutants with the ECM proteins, and the standarisation of techniques.

ABSTRACT

Streptococcus suis, a major pathogen of swine, is an emerging zoonotic agent which causes meningitis and septic shock. In this study, we investigated the ability of *S. suis* mutant Strain (SRTΔA) lacking the sortase A gene (*srtA*) to interact with host cells and extracellular matrix (ECM) proteins, as well as its virulence in a mouse infection model. We demonstrated that mutant SRTΔA had reduced capacity to adhere to and invade porcine brain microvascular endothelial cells compared to the wild-type strain. In addition, mutant SRTΔA also showed significantly less adherence to plasma fibronectin, cellular fibronectin and collagen type I. However, disruption of *srtA* had little effect on the virulence of *S. suis* in a mouse intraperitoneal model of infection. These results indicate that surface proteins anchored by sortase A are required for a normal level of bacterial binding. However, other factors may also be important for *S. suis* virulence and interaction with host tissues.

INTRODUCTION

Streptococcus suis serotype 2 is an important swine bacterial pathogen associated mainly with meningitis but also with other diseases such as endocarditis, arthritis, septicemia, and pneumonia (12). Among 35 described serotypes, *S. suis* serotype 2 is the serotype most frequently recovered from diseased animals (12). Recently, serotypes 32 and 34, unlike other serotypes, have been shown to be more related to *Streptococcus orisratti* (14). As a zoonotic agent, *S. suis* has been isolated from human cases of meningitis, endocarditis, and toxic shock-like syndrome (27, 29). A recent, unprecedented outbreak in China resulted in >200 human cases that were directly linked to a concurrent outbreak of *S. suis* infection in pigs. Of these human cases, 20% were fatal (29).

Our understanding of *S. suis* virulence factors remains limited. While a few factors have been shown to be critical for virulence, others are considered as putative although their precise role in *S. suis* related pathogenesis has yet to be clearly defined (11). In addition, the multistep pathogenesis of meningitis caused by *S. suis* is poorly understood (11). Most bacterial infections are initiated when bacteria adhere to host tissues. Likewise, initial interactions between *S. suis* and host tissues are also thought to be important for infection. In particular, interactions between *S. suis* and porcine brain microvascular endothelial cells (PBMEC) from the blood-brain barrier (BBB), which separates the central nervous system (CNS) from the bloodstream (10, 16), are thought to comprise a key step in the pathogenesis of *S. suis* induced meningitis (25).

The extracellular matrix (ECM) is a complex structural network beneath epithelial and endothelial cells and surrounding connective tissue cells (22). Recently, it has been established that *S. suis* binds to several immobilized serum and ECM proteins such as plasma and cellular fibronectins and collagen types I, III, and V. Moreover, the adhesin(s) responsible for these interactions are proteinaceous in nature (8). However, the mechanisms underlying interactions between these proteins and *S. suis* have not been elucidated. In other Gram positive bacteria, many cell surface proteins possessing a Leu-Pro-X-Thr-Gly (LPXTG) motif have been reported to mediate bacterial interaction with host cells, namely ECM, and thus play an important role in virulence (19). These specific proteins are covalently anchored to the cell wall by sortase A (SrtA), a membrane-bound thiol transpeptidase enzyme (19, 20). Five sortases of *S. suis* have been described thus far: SrtA, SrtB, SrtC, SrtD and SrtE (21). Of the five sortases, SrtA (recently classified as a class A sortase) has been shown to play a critical role in anchoring LPXTG proteins to *S. suis* cell wall (7, 21).

It is not clear whether SrtA and LPXTG proteins of *S. suis* are important for virulence and initial interactions between bacteria and host cells. In this study, we investigated the effects of a disruption of *srtA* gene on the capacity of *S. suis* to interact with PBMEC and ECM components and subsequently be virulent in an experimental mouse model of infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S. suis serotype 2 reference strain NCTC 10234 and its mutant SRTΔA, with targeted disruption of *srtA* as generated by allelic exchange via double crossover (21), were used in this study. Bacteria were grown overnight onto sheep blood agar plates at 37 °C and isolated colonies were incubated in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, MI, USA) for 8 h at 37 °C with agitation. Working cultures were obtained by inoculating 10 µl of a 10^{-3} dilution of these cultures in 30 ml of THB and incubating for 16 h at 37 °C with agitation. Bacteria were washed twice in phosphate buffered saline (PBS) pH 7.3, and were appropriately diluted in cell culture medium before infection. An accurate determination of the number of colony forming unit per ml (CFU/ml) in the final suspension was made by plating onto THB agar using Autoplate® 4000 (Spiral Biotech, Norwood, MA, USA). *S. suis* mutant SRTΔA was grown in the presence of 5 µg ml-1 of chloramphenicol (Sigma-Aldrich, Oakville, ON, Canada). Both strains showed similar growth rates (data not shown).

Cell culture

The PBMEC/C1-2 cell line (24)was cultivated as previously described (Vanier *et al.*, 2004). Briefly, cells were grown in a 1:1 mixture of Iscove's modified Dulbecco's medium and Ham's F-12 (Invitrogen, Burlington, ON, Canada) supplemented with 7.5% (v/v) heat inactivated fetal bovine serum, penicillin-streptomycin (Invitrogen), sodium bicarbonate, L-glutamine, human transferrin (MP Biomedicals, Solon, OH, USA), N-acetyl-cysteine, hypoxanthine, porcine heparin, human recombinant fibroblast growth factor-basic (Sigma-Aldrich), and β-mercaptoethanol (BioRad Laboratories, Mississauga, ON, Canada). Flasks (BD FalconTM; BD Biosciences, Mississauga, ON, Canada) and 24-well tissue culture plates (Primaria, BD FalconTM) were precoated with 1% (w/v) type A gelatin from porcine skin (Sigma-Aldrich). Cells were maintained at 37 °C with 5% CO2 in a humid atmosphere. For assays, PBMEC were trypsinized by adding a trypsin-EDTA solution (Invitrogen) and diluted in culture medium at 8 x 10^4 cells/ml. The cell suspension was distributed in tissue culture plates and incubated to confluence. Before the experiments, medium was removed from the plates and replaced by medium without antibiotics.

Adherence and invasion assays

For adhesion assay, bacteria were pelleted, washed twice with PBS, and resuspended at 10^6 CFU/ml in fresh cell culture medium without antibiotics as previously described (25, 26). Confluent cell monolayers were infected with 1 ml aliquots of bacterial suspension. Plates were centrifuged at

800 × *g* for 10 min and incubated for 2 h at 37 °C with 5% CO2. The monolayers were washed five times with PBS, and incubated with 200 µl of 0.05% trypsin-0.03% EDTA for 10 min at 37 °C. After the addition of 800 µl of ice-cold deionised water, cells were detached and disrupted by scrapping the bottom of the well. Serial dilutions of this cell lysate were plated onto THB agars to enumerate viable bacteria. An invasion assay to quantify intracellular bacteria was performed in a similar manner as the adhesion assay, with one exception. Following the initial infection period, cells were washed twice with PBS and incubated with 1 ml of cell culture medium containing 100 µg/ml of gentamicin and 5 µg/ml of penicillin G (Sigma-Aldrich) for 1 h at 37 °C with 5% CO2 (Vanier et al., 2004; Vanier et al., 2007). Levels of adhesion and invasion are expressed as the total number of CFU recovered per well.

Binding of *S. suis* to ECM proteins

Bacteria grown to a late exponential-phase were washed three times in PBS, resuspended at a concentration of 10^8 CFU/ml in 0.2% (v/v) formaldehyde, and incubated overnight at 4 °C. An enzyme linked immunosorbent assay (ELISA) was performed as previously described (8) using microtiter plates (Maxisorp, Nunc, VWR, Mississauga, ON, Canada) that had been coated overnight at 4 °C with 100 µl of plasma fibronectin (1 to 10 µg/ml) (Roche Diagnostics Corp., Indianapolis, IN, USA), cellular fibronectin (20 µg/ml) (Sigma-Aldrich), or collagen type I (1 to 12.5 µg/ml) (BD Biosciences) in 0.1 M carbonate coating buffer (pH 9.6). Wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST, pH 7.3), and blocked with 200 µl of 3% (w/v) non-fat dry milk in PBST. After 1 h at 37 °C, wells were washed three times with PBST and formaldehyde-killed bacterial suspensions (100 µl) were added. Plates were incubated for 2 h at 37 °C and washed three times. A 100 µl volume of *S. suis* serotype 2-specific rabbit antiserum (13) (diluted 1/3000 in PBST) was then added to each well. Plates were incubated for 1 h at 37 °C and were washed three times. A volume of 100 µl of horseradish peroxidase-labelled anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) (diluted 1/8000 in PBST) was added and plates were incubated for 1 h at 37 °C. After washing three times with PBST, 3,3',5,5'-TetraMethylBenzidine (Zymed, Invitrogen) was used according to the manufacturer's instructions. Reactions were stopped by adding 25 µl of H2SO4 (1 N) and then read at 450 nm using a microplate reader (UVmax; Molecular Devices, Sunnyvale, CA, USA). Uncoated and casein-coated wells served as background and non-specific adhesion of *S. suis* to protein coated control wells, respectively. In addition, controls were performed to ensure that *S. suis* serotype 2- specific rabbit antiserum recognized wild-type strain and SRTΔA equally.

Intraperitoneal mouse virulence model

A total of 40 female CD1 6-week-old mice (Charles River Laboratories, Wilmington, MA, USA, 10 animals per group) were infected intraperitoneally with either 1 ml of *S. suis* strain NCTC 10234 or 1 ml of mutant SRTΔA (approximately 108 CFU in PBS). The CD1 mouse model was recently shown to be an excellent model of *S. suis* infection causing septic shock and meningitis (6). Body weight changes (due to an excessive production of pro-inflammatory cytokines) were recorded daily post-infection (p.i.) (6). Survival and development of clinical signs of meningitis were recorded over a 10-day period. In addition, bacterial numbers in blood and homogenized brain samples were determined by plating on sheep blood agar plates using Autoplate® 4000 at different timepoints p.i . Experiments involving mice were repeated twice (total number of 20 animals per group) and 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, Animal Welfare Committee of the Université de Montréal.

Statistical analysis

All data are expressed as means τ standard deviations (error bars). Data were analyzed by twotailed, unpaired *t* test. Unless specified, all assays were repeated at least three times. Statistical analysis of the survival data was performed with the LogRank test. A *P* value of ≤ 0.05 was accepted as the minimal level of significance.

RESULTS

srtA gene contributes to the interactions between S. suis and endothelial cells

As shown in Fig. 1, adhesion and invasion levels of mutant SRTΔA were strongly reduced to 16 ± 24% and 17 ± 12%, respectively, compared to those of the wild-type strain (considered as 100%) when 10^6 CFU ml-1 bacterial suspensions were used. Similar results were obtained with different concentrations of bacteria (data not shown). The complemented mutant SRTΔA (Osaki et al., 2002) showed adhesion and invasion values of 88 ± 17% (P = 0.008 compared to the mutant SRTΔA) and 47 ± 4% (P = 0.004 compared to the mutant SRTΔA), respectively. These results confirm that sortase A is at least partially involved in *S. suis*-specific adhesion to and invasion of endothelial cells.

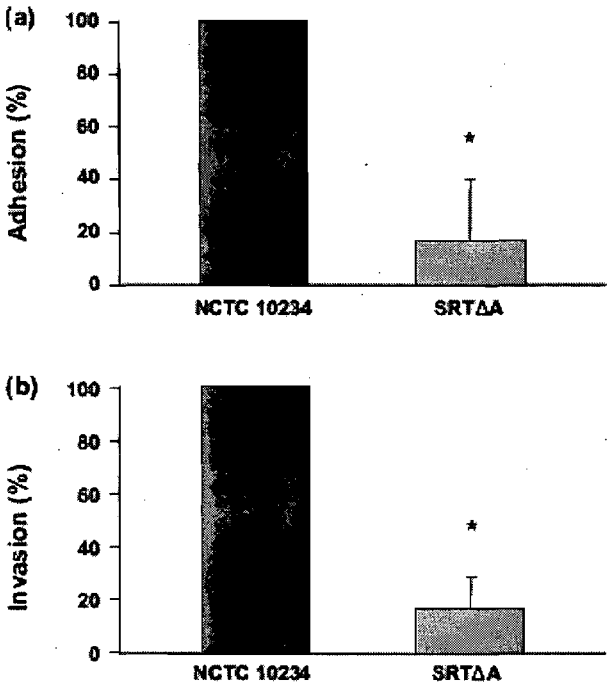

Fig. 1. (a) Adhesion to PBMEC by mutant SRTΔA compared to the wild-type strain NCTC 10234. (b) Invasion of PBMEC by mutant SRTΔA compared to the wild-type strain NCTC 10234. Adhesion and invasion are expressed as a percentage of the adhesion and invasion levels achieved by the wild-type strain NCTC 10234. Asterisk denotes values that are significantly different ($P < 0.05$) from those of NCTC 10234.

***srtA* gene contributes to adherence of *S. suis* to ECM proteins**

An ELISA binding assay was used to determine whether the mutant SRTΔA had altered capacity to bind plasma and cellular fibronectins and collagen type I. As shown in Fig. 2 (a), SRTΔA had reduced binding to plasma fibronectin by ($P < 0.05$) compared to the wild-type strain. A similar decrease in adhesion to cellular fibronectin was also observed (data not shown). Binding to collagen type I by mutant SRTΔA was also diminished ($P < 0.05$; Fig. 2 (b)). Adhesion to fibronectin and collagen type I by both strains was dependent on the protein concentration (Fig. 2).

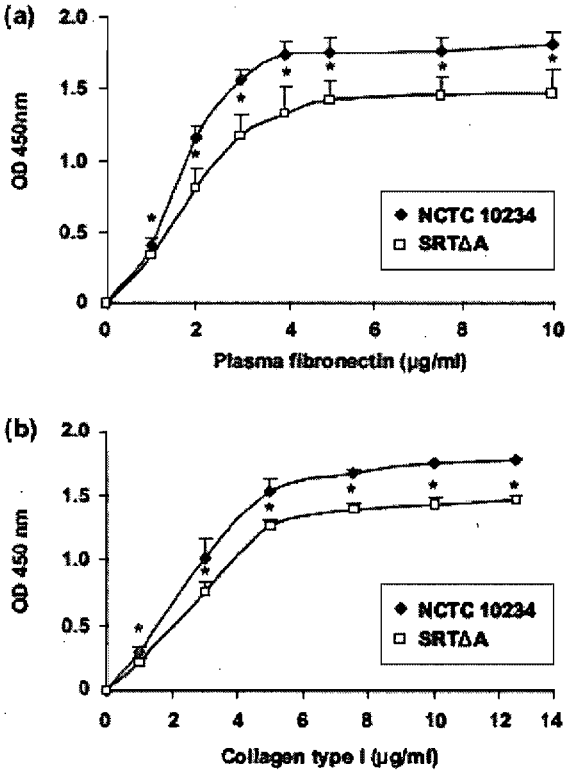

Fig. 2. Binding to different concentrations of plasma fibronectin (a) and collagen type I (b) by mutant SRTΔA compared to the wild-type strain NCTC 10234. Asterisk denotes values that are significantly different ($P < 0.05$) between the two strains.

Effect of *srtA* gene disruption on virulence

Animals from both groups showed only limited weight loss (0 to 10% of initial weight) between day (d) 0 and d 5, with maximum loss observed at d 2 p.i. (data not shown). In addition, weight gain from d 6 to the end of the experiment was not significantly different between groups ($P > 0.05$; data not shown). With respect to cumulative survival, CD1 mice infected with SRTΔA had slightly longer median survival (2 d), although this was not statistically significant ($P > 0.05$)

compared to mice infected with the wild-type strain (1 d) (Fig. 3). Both strains displayed a similar mortality rate, with 80% of all animals dying within 10 d. Morbidity also did not differ between the groups; one mouse in each group developed clinical signs of meningitis (i.e. spatial disorientation). Blood and brain samples collected from infected animals during the first 2 d p.i. showed no significant differences in bacterial load between the two groups (data not shown).

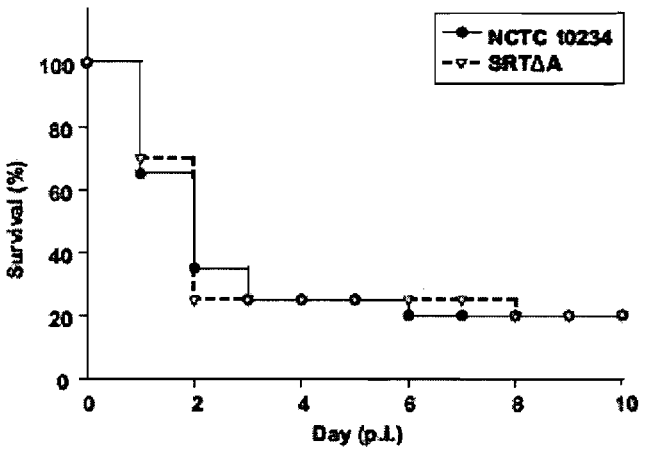

Fig. 3. Kaplan-Meier survival analysis of CD1 mice infected with the wild-type strain NCTC 10234 or the mutant SRTΔA (P = 0.56 for survival times between both groups). Six-week-old CD1 mice were inoculated i.p. with 108 bacteria, and survival was monitored over a 10-day period. Data are expressed as mean percentage of live animals in each group (n=20).

DISCUSSION

In this study, we demonstrated that the mutant SRTΔA strain of *S. suis* has markedly impaired interactions with PBMEC *in vitro*. These findings provide evidence that sortase A of *S. suis* is involved in adhesion to and invasion of host cells and are consistent with those reported for other Gram-positive pathogens, such as *Streptococcus pneumoniae* (15), *Streptococcus sanguinis* (28), and *Streptococcus agalactiae* (17). Our results also showed mutant SRTΔA has reduced binding to ECM proteins, suggesting that SrtA of *S. suis* may anchor LPXTG-containing microbial surface components which recognize adhesive matrix molecules (MSCRAMM) that mediate binding to plasma and cellular fibronectins and to collagen type I. These findings are in agreement with previous studies on *S. agalactiae* (17) and *S. gordonii* (3), showing that SrtA-expressing strains bind in significantly higher numbers to fibronectin compared to isogenic *srtA* mutant strains. Together these results suggest that LPXTG proteins anchored by SrtA are at least partially involved in the interactions between *S. suis* and host tissues.

Few LPXTG proteins of *S. suis* have been identified. Among these, the opacity factor of *S. suis* (OFS) and surface antigen one (Sao) have been recently described (1, 18). OFS is an opacity factor involved in virulence but not in colonization (1). Sao is an immunogenic protein that is expressed on the bacterial surface and confers protection to immunized animals (18). Moreover, surface expression of some LPXTG proteins is downregulated after disruption of the *srtA* gene (21). Indeed, mutant SRTΔA of *S. suis* has reduced expression of more than 15 major protein spots as visualized with two-dimensional polyacrylamide gel electrophoresis. From these protein spots, a previously described protein, the muramidase-released protein (MRP), as well as other newly described proteins similar to 5'-nucleotidase, and encoded by the genes *sntA*, *sntB*, and *sntC*, were identified. In European *S. suis* strains, MRP is considered to be a virulence marker (11). However, MRP alone is not necessary for virulence in swine and is absent in most North American virulent strains (12). Other putative *S. suis* LPXTG proteins and their role in virulence have yet to be identified. Moreover, the precise LPXTG protein(s) involved in *S. suis*-host interactions are unclear and warrant further investigation.

It should be noted that a deficiency of sortase A did not completely abolish interactions between *S. suis* and PBMEC or ECM proteins, suggesting that other factors may also contribute to *S. suis* mediated pathogenesis. This is in agreement with our previous observations (26) demonstrating that non-proteinaceous cell wall components, such as lipoteichoic acid (LTA), are also involved in interactions between *S. suis* and PBMEC. Other pathogens, such as *S. pyogenes*,

have been shown to possess multiple types of adhesins (LTA and proteins) that mediate interactions with host cells (4). Non-LPXTG proteins (non-MSCRAMM) might also be involved in binding to ECM proteins. Notably, *S. suis* possesses non-MSCRAMM ECM-binding proteins such as FBPS (5), a 52-kDa IgG-binding protein (23), and a recently described Enolase (9). In a previous study, bacteria coated with plasma fibronectin showed high levels of adhesion to and invasion of PBMEC, suggesting that *S. suis* uses plasma fibronectin as a bridge between bacteria and the host cell surface (26). Results obtained in the present study suggest that non-MSCRAMM as well as LPXTG-containing MSCRAMM ECM-binding proteins may be involved in these host-pathogen interactions.

Disruption of *srtA* gene had little effect on the virulence of *S. suis* in a mouse intraperitoneal model of infection. Our findings are consistent with those reported by Kharat and Tomasz (2003) showing that a *srtA* mutant of *S. pneumoniae* was as virulent as the wild-type strain in mice. Paterson and Mitchell (2006) demonstrated using a competitive infection model that *srtA* is a factor required for fitness rather than virulence of *S. pneumoniae*. It is not known whether a similar function can be attributed to *srtA* in *S. suis* infection. Indeed, there were no significant differences in levels of bacteremia between the wild-type and mutant strains. Survival of bacteria in blood is a critical step that enables the bacteria to disseminate throughout the host and ultimately gain access to the CNS. There is much evidence that the capsule is an important virulence factor that promotes bacterial resistance to phagocytic clearance (11). Recent results in our laboratory obtained using a coagglutination assay showed that the mutant SRTΔA is encapsulated (unpublished observation), which may explain its normal bacterial loads in blood. A high-grade bacteraemia was shown to be required for *S. suis* to subsequently reach the CNS, thus suggesting a critical role of the capsule for bacterial survival and dissemination in blood during *S. suis* invasion of the BBB (2).

Lastly, other class C sortases genes (*srtB*, *srtC*, *srtD* and *srtE*) (7) may also contribute to *S. suis*-host interactions. In *S. suis* NCTC 10234 strain, three class C sortases genes (*srtB*, *srtC*, *srtD*) cluster together and are associated with LPXTG proteins (21). It is possible that these accessory class C sortases, which are present in about 50% of virulent *S. suis* strains (T. Sekizaki, unpublished observations), might specifically anchor the flanking LPXTG-containing proteins. Studies are ongoing in our laboratory to verify the role of other *S. suis* sortases in bacterial interactions with host cells and proteins.

We conclude that *S. suis* SrtA anchors LPXTG-containing surface proteins, thus playing an important role in bacterial colonization of host cells and adhesion to ECM proteins. However,

anchorage of LPXTG proteins alone is not sufficient for virulence in mice. Together, these observations suggest that although LPXTG-containing surface proteins participate in multiple steps of the pathogenesis of *S. suis* infection, other non-LPXTG proteins, including secreted, membrane-bound and non-proteinaceous components, are likely to be involved in cell colonization, ECM binding, and virulence.

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