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IDENTIFICATION AND CHARACTERIZATION OF NOVEL VIRULENCE FACTORS FROM THE SWINE PATHOGEN AND ZOONOTIC AGENT *STREPTOCOCCUS SUIS*

par

NAHUEL FITTIPALDI

Département de pathologie et microbiologie

Faculté de médecine vétérinaire

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présentée par

NAHUEL FITTIPALDI

a été évaluée par un jury composé des personnes suivantes :

Mariela Segura, présidente-rapporteuse

Marcelo Gottschalk, directeur de recherche

Josée Harel, codirectrice

Charles Dozois, membre du jury

Michel Frenette, examinateur externe

John Fairbrother, représentant du doyen de la FÉSP

RESUMÉ

Streptococcus suis est un pathogène du porc et un agent de zoonose causant, entre autres, une septicémie et une méningite tant chez le porc que chez l'humain. Le sérotype 2 est considéré comme étant le plus virulent des trente cinq sérotypes de *S. suis*. La pathogenèse de l'infection à *S. suis* demeure mal connue et, pour la plupart, les connaissances sont limitées au sérotype 2. De plus, peu des facteurs de virulence proposés se sont avérés être essentiels pour la virulence de ce pathogène. Pour causer la méningite *S. suis* doit atteindre le système nerveux central (CNS), ce qui implique traverser les barrières hémato-méningée et/ou hémato-encéphalique (BBB). Les cellules endothéliales de microvaisseux cérébraux (BMEC) sont des composants majeurs de la BBB. En utilisant un modèle d'infection *in vitro* comportant des BMEC porcines immortalisées, il a été démontré que *S. suis* est capable de s'attacher et d'envahir ces cellules. Nous proposons que les gènes qui sont exprimés différentiellement par *S. suis* lors de ses interactions avec les BMEC porcines pourraient potentiellement être des facteurs de virulence importants de ce pathogène. Afin de déterminer l'expression génique de la bactérie lors de l'interaction *S. suis*-BMEC porcines et évaluer cette hypothèse, nous avons utilisé la méthode de capture sélective de séquences transcrites (SCOTS). L'étude a permis l'identification de vingt-huit gènes différentiellement exprimés par *S. suis* lors des processus d'adhésion/invasion de ces cellules formant la BBB. Certains de ces gènes ont présenté un clair potentiel comme facteurs de virulence. Cinq d'entre eux sont putativement impliqués dans la modification de composants de la paroi cellulaire tels que les acides lipotéichoiques (LTA) et le peptidoglycane. De plus, l'utilisation de SCOTS a permis l'identification, pour la première fois chez *S. suis*, d'un locus dont les gènes codent pour un pilus. En effet, un gène isolé par SCOTS a été reconnu comme codant pour une peptidase signal étant le premier gène dudit locus. D'autres gènes identifiés par SCOTS codent putativement pour différentes protéines de la paroi cellulaire, pour des transporteurs, pour des régulateurs transcriptionels, pour des gènes de ménage ainsi que pour des protéines à fonction inconnue.

Nous avons effectué des études additionnelles afin de comprendre la contribution de la production de pili à la virulence de *S. suis*. Des mutants pour chacun des gènes du locus piliare ont été construits. De plus, nous avons produits des anticorps dirigés contre les sous-unités piliaires. Les résultats préliminaires ont montré qu'une souche hautement virulente produit un pilus, lequel est toutefois dépourvu d'adhésine. L'abolition de l'expression de ce pilus sans

adhésine n'a pas altéré les interactions de *S. suis* avec les BMEC porcines. De plus, un mutant non-pilié a été aussi virulent que la souche sauvage (WT) dans un modèle murin de l'infection. Ces résultats suggèrent que la production de pili n'est pas essentielle pour la virulence de *S. suis*.

D'autres gènes identifiés par SCOTS ont aussi été caractérisés. Les gènes ciblés ont été *dltA*, lequel est impliqué dans D-alanylation des LTA, une modification résultant dans une stabilisation de la paroi cellulaire par neutralisation des charges négatives chez certains pathogènes à Gram positifs; et *pgdA*, dont le rôle est la N-deacétylation du peptidoglycane, une modification qui, chez d'autres pathogènes, a comme conséquence une résistance accrue face à l'action hydrolytique du lysozyme. Pour évaluer davantage la contribution du gène *dltA* à la pathogénèse de l'infection à *S. suis*, un mutant Δ*dltA* a été construit chez une souche hautement virulente de sérotype 2. L'analyse par résonance magnétique nucléaire a prouvé que les LTA du mutant étaient dépourvus de D-alanine, confirmant le phénotype escompté. L'absence de D-alanylation des LTA a eu comme conséquence une susceptibilité augmentée face à l'action des peptides antimicrobiens cationiques. En outre, et contrairement à la souche WT, le mutant Δ*dltA* a été efficacement tué par des neutrophiles porcines et a montré des interactions diminuées avec des BMEC porcines. Finalement, de par sa capacité diminuée à échapper aux mécanismes immunitaires innés de l'hôte et à traverser des barrières, le mutant Δ*dltA* a été atténuée dans les modèles murin et porcin d'infection.

Pour analyser la contribution du gène de *pgdA* à la virulence de *S. suis* nous avons déterminé, pour la première fois, la structure fine de son peptidoglycane en utilisant la chromatographie en phase liquide de haute performance et la spectrométrie de masse. Le peptidoglycane de *S. suis* s'est avéré être formé par vingt muropeptides, y compris trois qui sont N-deacétylés. La comparaison avec un mutant Δ*pgdA* a montré que le produit du gène *pgdA* est nécessaire pour cette modification spécifique, laquelle est produite en très faible quantité. Le bas niveau de N-deacétylation du peptidoglycane s'est corrélé avec une absence de résistance face au lysozyme *in vitro* par la souche WT. Cependant, l'expression du gène *pgdA* a été fortement régulée à la hausse lors de l'interaction de *S. suis* avec des neutrophiles porcins aussi qu'*in vivo* chez des souris inoculées expérimentalement, suggérant que *S suis* pourrait augmenter le niveau de N-deacétylation de son peptidoglycane dans ces conditions. De par sa capacité réduite à persister dans le sang, le mutant Δ*pgdA* a été sévèrement atténuée dans les modèles murins et porcins d'infection.

En conclusion, ce projet de recherche fournit des éléments originaux au sujet de la pathogenèse de l'infection à *S suis*. Nous avons montré que la D-alanylation des LTA et la N-deacétylation du peptidoglycane sont des mécanismes importants employés par ce microbe pathogène pour contrer les défenses de l'hôte. Les résultats présentés ici, aussi que la future caractérisation d'autres facteurs de virulence candidats identifiés par SCOTS pourrait s'avérer d'importance pour réduire le fardeau des maladies causées par *S. suis*.

Mots clés: *Streptococcus suis*, facteurs de virulence, pili, D-alanylation des LTA, N-deacétylation du peptidoglycane, capture sélective de séquences transcrites

SUMMARY

Streptococcus suis is a pathogen of swine and a zoonotic agent responsible for, among other diseases, meningitis and septicemia in both swine and humans. The pathogenesis of the *S. suis* infection remains poorly known. Indeed, knowledge is limited to the serotype 2, which has been shown to be the most virulent of the thirty-five serotypes described so far. Only a few virulence factors have been proposed and less shown to be essential for the virulence of this pathogen. To cause meningitis, *S. suis* must cross the blood brain barrier (BBB) and gain access to the central nervous system (CNS). Brain microvascular endothelial cells (BMEC) are one of the main BBB-forming cellular types. Using an *in vitro* model of infection *S. suis* has proven able to attach to and to invade immortalized porcine BMEC. We hypothesized that genes preferentially expressed by *S. suis* during its interactions with these cells may constitute potential important virulence factors of this swine pathogen and zoonotic agent. Therefore, we used the selective capture of transcribed sequences (SCOTS) approach to dissect bacterial gene expression upon the interactions of *S. suis* with porcine BMEC. The study led to the identification of twenty-eight genes that the pathogen preferentially expressed during attachment/invasion of these BBB-forming cells. Five of the identified genes were shown to have putative roles in modification of cell wall structures such as the lipoteichoic acid (LTA) and the peptidoglycan. Some of these genes presented a clear potential as virulence factors. In addition, the use of SCOTS led to the identification, for the first time, of a pilus cluster in *S. suis*. Indeed, one gene encoding a putative signal peptidase was isolated which was found to the first gene of that pilus cluster. Other identified genes putatively encoded different cell wall proteins, transporters, transcriptional regulators, housekeeping genes and proteins of unknown function.

We performed additional studies in order to understand the contribution of pili production to the virulence of *S. suis*. Characterization of the pilus cluster identified by SCOTS included the construction of mutant strains for each member of the cluster as well as the generation of antibodies against the putative pilin subunits. Preliminary results revealed that a highly virulent *S. suis* serotype 2 strain indeed produces a pilus, which is, nevertheless, devoid of the putative adhesin subunit. Interestingly, abolition of the expression of this adhesin-less pilus did not result in impaired interaction with porcine BMEC. Furthermore, a non-piliated mutant of a highly virulent serotype 2 strain was as virulent as the wild type (WT) strain when evaluated in

a murine model of infection. These results might suggest that pili may be dispensable for the virulence of *S. suis.*

Other SCOTS identified genes,*dltA* and *pgdA*, were further characterized. The *dltA* gene is involved in LTA D-alanylation, a modification resulting in cell stabilization by neutralization of negative charges in some Gram positive pathogens. The *pgdA* gene plays a role in the N-deacetylation of peptidoglycan, a modification shown in other pathogens to result in enhanced resistance to the action of lysozyme. To further evaluate the contribution of the *dltA* gene to the pathogenesis of *S. suis* infection, a Δ*dltA* mutant strain was generated in a highly virulent serotype 2 strain. Nuclear magnetic resonance showed that the LTAs of the mutant were devoid of D-alanine, confirming the expected phenotype. Absence of LTA D-alanylation resulted in an increased susceptibility to the action of cationic antimicrobial peptides. In addition, and in contrast to the WT strain, the Δ*dltA* mutant was efficiently killed by porcine neutrophils and showed impaired interactions with porcine BMEC. Finally, reflecting decreased ability to escape immune clearance mechanisms and reduced capacity to traverse across host barriers, the Δ*dltA* mutant was attenuated in both the murine and porcine models of infection.

To analyze the contribution of the *pgdA* gene to the virulence of *S. suis*, we determined for the first time the fine structure of *S. suis* peptidoglycan using high performance liquid chromatography and mass spectrometry. The *S. suis* peptidoglycan was found to contain twenty muropeptides, including three that are N-deacetylated. Comparison with a Δ*pgdA* mutant showed that the *pgdA* gene product was required for this specific modification, which occurred in very low amounts. Interestingly, the low level of peptidoglycan N-deacetylation correlated with absence of significant lysozyme resistance when WT *S. suis* was grown *in vitro*. However, expression of the *pgdA* gene was highly upregulated upon interaction with porcine neutrophils as well as *in vivo* in experimentally inoculated mice, suggesting that *S. suis* may enhance peptidoglycan N-deacetylation under these conditions. Reflecting a decreased ability to persist in blood, the Δ*pgdA* mutant was severely attenuated in both the murine and porcine models of infection.

In conclusion, this research has provided novel insights about the pathogenesis of *S. suis*. We showed that both LTA D-alanylation and peptidoglycan N-deacetylation are important mechanisms used by this pathogen to successfully counter the host defenses. Results presented

here, as well as future characterization of other virulence factor candidates identified by SCOTS may be of importance to reduce the burden of *S. suis* disease.

Keywords: *Streptococcus suis*, virulence factors, pili, LTA D-alanylation, peptidoglycan N-deacetylation, selective capture of transcribed sequences

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LIST OF ABBREVIATIONS

A_2pm.....2, 6-diaminopimelic acid

ADS.....Arginine deiminase system

AI ..... Acquired islands/ Autoinducer

BBB ..... Blood-brain barrier

BMEC.. Brain microvascular endothelial cells

CAMPs ..... Cationic antimicrobial peptides

CDS .....Coding sequence

CNS ..... Central nervous system

CPEC ..... Choroid plexus epithelial cells

CPS ..... Capsular polysaccharide

CSF.....Cerebrospinal fluid

CWG ..... Cell wall glycopolymer

DNA .....Deoxyribonucleic acid

DPD 4, 5-dihydroxy-2, 3-pentanedione

DPP.....Dipeptidyl peptidase

ECM .....Extracellular matrix

EF.....Extracellular protein factor

Ffbp.....Fibronectin/fibrinogen binding protein

GAG .....Glycoaminoglycan

Gal ..... Galactose

GalNAc .....N-acetyl galactosamine

GAPDH..... Glyceraldehyde-3-phosphate dehydrogenase

GAS..... Group A Streptococcus

GBS..... Group B Streptococcus

GDH .....Glutamate dehydrogensase

Glc ..... Glucose

GlcNAc.....N-acetyl glucosamine

Gro-P .....Glycerol phosphate

HNP ..... Human neutrophil peptide

IFN ..... Interferon

IgG .....Immunoglobulin G

IL..... Interleukin

ISR..... Intergenic space region

IVET ..... *In vivo* expression technology

Ka.....Nonsynonymous substitutions

KC ..... Keratinocyte-derived chemokine

Ks..... Synonymous substitutions

LI ..... Lost islands

LTA.....Lipoteichoic acid

MCP .....Monocyte chemotactic protein

MLEE..... Multilocus enzyme electrophoresis

MLST.....Multilocus sequence typing

MRP .....Muramidase-released protein

MSCRAMM.....Microbial surface components recognizing adhesive matrix molecules

MurNac.....N-acetyl muramic acid

NET ..... Neutrophil extracellular traps

NeuNAc .....N-acetyl neuraminic acid

NF .....Nuclear factor

OCT..... Ornithine carbamoyl-transferase

OFS ..... Serum opacity-like factor

ORF ..... Open reading frame

PAGE.....Polyacrylamide gel electrophoresis

PAI ..... Pathogenicity island

PCR ..... Polymerase chain reaction

PCV ..... Porcine circovirus

PEP..... Phosphoenolpyruvate

PFGE .....Pulse field gel electrophoresis

PRR ..... Pattern-recognition receptor

PRRS.....Porcine reproductive and respiratory syndrome

PTS.....Phospho transferase system

Q-RT-PCR.....Quantitative-Reverse transcription-Polymerase chain reaction

RANTES.....Regulated upon activation, normal T cell expressed and secreted

RAPD.....Randomly amplified polymorphic DNA

rDNA..... Ribosomal RNA-encoding DNA

RFLP.....Restriction fragment length polymorphism

Rha ..... Rhamnose

RNA ..... Ribonucleic acid

RT-PCR.....Reverse transcription-Polymerase chain reaction

SAH .....S-adenosylhomocysteine

SAM .....S-adenosylmethionine

SCOTS.....Selective capture of transcribed sequences

SDS ..... Sodium dodecyl sulfate

SNP .....Single nucleotide polymorphism

SOD..... Superoxide dismutase

ST..... Sequence type

STM .....Signature-tagged mutagenesis

STSS ..... Streptococcal toxic shock syndrome

TLR..... Toll-like receptor

TNF .....Tumor necrosis factor

US ..... United States

WT ..... Wild-type

WTA.....Wall teichoic acid

A mi familia

y

A Pedro C. Micale, in memoriam.

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I. INTRODUCTION

Streptococcus suis is an important swine pathogen responsible for a wide range of diseases in swine, the most striking of which are septicemia and meningitis. In addition to causing disease in pigs, *S. suis* is an emerging zoonotic agent. Indeed, this pathogen is increasingly becoming a public health concern in several Asian countries, such as Vietnam, Thailand, Hong Kong and China. For instance, during a single outbreak in 2005 in that latter country, more than two hundreds cases of human *S. suis* disease were reported, thirty-nine of which resulted in death. Of the thirty-five *S. suis* serotypes that have been described, the serotype 2 is considered to be the most virulent. It is also the most prevalent serotype isolated from diseased pigs in the majority of countries where the swine production is important. In pigs, S. *suis* is mainly transmitted via the respiratory route and remains localized in the tonsils. In fact, some animals may remain healthy carriers for life (they will never develop disease), while, on the other hand, some animals may eventually develop bacteremia, sometimes septicemia (fulminant systemic disease), and/or meningitis.

Multiple lines of evidence suggest that both the pathogen, through the production of virulence factors, and the host, through an exacerbated immune response to the infection, are responsible for the pathology of *S. suis* diseases in both swine and humans. The understanding of the contribution of the host immunity to the pathogenesis of *S. suis* infection has significantly advanced in recent years. However, despite increasing research, knowledge on *S. suis* virulence factors remains limited. *S. suis* is an encapsulated bacterium and it has been demonstrated that the capsular polysaccharide (CPS) confer antiphagocytic properties. In fact, presence of the CPS is necessary for persistence of the organism in blood, a very important condition that must be met for the development of septicemia and meningitis. However, the CPS is not sufficient for survival in this tissue as shown by the fact that well encapsulated avirulent strains are rapidly cleared from circulation. Indeed, most avirulent *S. suis* strains are encapsulated. Recently, a serum opacity-like factor (OFS) has been shown to play a critical role in the pathogenesis of the *S. suis* infection. However, the OFS seems to be absent from many virulent isolates. As well, many, but not all, *S. suis* virulent strains secrete a hemolysin (known as suilysin, a thiol-activated toxin) which may contribute to the virulence of the organism. However, isogenic mutants lacking suilysin have been shown to be as virulent as the parent strain after experimental infection of pigs. Besides, as stated above, some virulent strains do not produce suilysin. Other proposed putative virulence factors include the extracellular

protein factor (EF) and the muramidase-released protein (MRP). These factors have been shown to be associated to virulence, but several studies using isogenic mutants have shown that they are not essential for the full virulence of the organism. In addition, as is the case for suilysin, these protein factors are absent from many virulent strains. Other determinants, such as a fibronectin/fibrinogen-binding protein (Ffbp), have been found to be only partially involved in virulence. Finally, the actual role of some other virulence candidates (several putative adhesins and proteases) in the pathogenesis of *S. suis* infection remains to be verified. Interestingly, with the exception of the CPS, extracellular structures of the pathogen, such as cell wall components, have not been found to play a direct role in virulence, although they have been shown to very much contribute to the exacerbation of the host inflammatory response.

As stated above, *S. suis* causes meningitis. A prerequisite for the onset of this disease is pathogen invasion of the central nervous system (CNS). The actual mechanisms by which *S. suis* gains access to the CNS remain so far unknown. An early hypothesis, known as the "Trojan horse", suggested uptake of bacteria by monocytes, intracellular survival and invasion of the CNS. However, results of several different studies suggest that this mechanism is unlikely to be important for CNS invasion. Indeed, as a result of these studies, a "modified Trojan horse" hypothesis has been put forward, in which the pathogen, rather than being phagocytosed, establishes an extracellular association with monocytes/macrophages and takes advantage of the infiltration of these cells to break into the CNS. It has also been suggested that the pathogen might reach the CNS by disrupting or by crossing without disruption the blood brain barrier (BBB) and/or the blood-cerebrospinal fluid (CSF) barrier. Indeed, *in vivo* (murine model of infection) studies suggest that the pathogen may gain access to the CNS through the brain microvascular endothelium and, also, through the choroid plexus epithelium. Further support for these mechanisms has been provided by recent reports showing that *S. suis* is able to affect the viability of porcine choroid plexus epithelial cells (CPEC) composing the blood-CSF barrier through necrotic and apoptotic mechanisms. In addition, invasion of BBB-forming cells such as brain microvascular endothelial cells (BMEC) has been demonstrated using an *in vitro* model of cultured porcine BMEC.

Although further studies are needed to definitively ascertain how the pathogen enters the CNS, the development of the latter model of *S. suis*-porcine BMEC interactions offers a

unique opportunity to elucidate the weapons this pathogen makes use of during a critical step for the development of *S. suis* meningitis such as BBB crossing. Indeed, it can be hypothesized that genes expressed by *S. suis* during its interactions with porcine BMEC may potentially constitute important virulence factors of this swine pathogen and zoonotic agent.

The main objective of this research project is to increase the current understanding of the pathogenesis of *S. suis* by the identification and characterization of novel virulence factors of this organism. It is divided in two specific objectives:

1. The first specific objective is the determination of genes differentially expressed by *S. suis* during its interactions with porcine BMEC and the identification of putative virulence factor candidates. The first specific objective takes advantage of the technological advance of the selective capture of transcribed sequences (SCOTS), which is a state-of-the-art, PCR-based RNA analysis method that directly identifies bacterial genes rather than promoter regions and is not confounded by polar effects when genes are arranged in polycistronic operons.
2. The second specific objective is the characterization of the actual contribution to the virulence traits of *S. suis* of selected SCOTS-identified putative virulence factors. Since the vitro model used for gene identification in specific objective 1 is inherently limited and, on the other hand, the SCOTS-identified genes may eventually also contribute to the virulence traits of *S. suis* at other stages of the infection, selected identified genes will be inactivated in a virulent strain and the resulting mutants tested in different experimental *in vitro* and *in vivo* models of infection.

II. REVIEW OF THE LITERATURE

1. *Streptococcus suis*

S. suis is a swine pathogen responsible for important losses to the porcine industry worldwide. This organism can produce a wide range of diseases, including meningitis, arthritis, pneumonia, septicemia, endocarditis and polyserositis (Higgins and Gottschalk, 2006). *S. suis* is also a zoonotic agent causing meningitis, septicemia, toxic shock-like syndrome and endocarditis in humans (Gottschalk et al., 2007). *S. suis* (Figure 1) is a Gram-positive small non-motile ovoid coccus, possessing cell wall antigenic determinants related to Lancefield group D, although it is genetically unrelated to other members of this group (Higgins and Gottschalk, 2006). *S. suis* is less than 2 μm in diameter, it occurs singly, in pairs and, rarely, in short chains. All strains are α-hemolytic on sheep blood agar and many produce β-hemolysis on horse blood agar. *S. suis* is chemo-organotroph with fermentative and facultative anaerobic metabolisms (Higgins and Gottschalk, 2006). Different biochemical tests have been used for the identification of *S. suis* and biochemical variations have been noted among different strains (Higgins and Gottschalk, 1990; Tarradas et al., 1994). However, a minimal number of biochemical tests carried out in combination with serotyping can allow for the definitive identification of most *S. suis* isolates recovered from pigs. The use of at least four tests has been proposed for a presumptive identification of S. *suis:* absence of growth in 6.5 % NaCl agar, a negative Voges-Proskauer test, production of acid in trehalose and salicin broth and production of amylase but not acetoin (Devriese et al., 1991; Higgins and Gottschalk, 1990). The NaCl (6.5%) test clearly differentiates between S. *suis* and *Streptococcus bovis* from the genus *Enterococcus.* The Voges-Proskauer test is critical and the most reliable for differentiating S. *suis* from S. *bovis* (Higgins and Gottschalk, 1990).

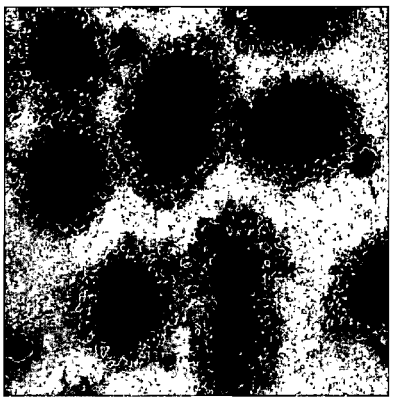

Figure 1. Transmission electron micrography of *S. suis* serotype 2 reference strain S735.
The ovoid coccus morphology and the thick capsule surrounding the pathogen are clearly visible. Magnification: 20,000 X. Source : Fittipaldi et al, unpublished results.

1.1. Diagnosis

Presumptive diagnosis of *S. suis* infections is based on clinical signs and macroscopic lesions. Diagnosis is confirmed by the isolation of the infectious agent and the recognition of microscopic lesions in tissues. Direct microscopy from affected organs or blood may confirm the streptococcal etiology of the disease. Isolation of *S. suis* from lungs has to be nevertheless interpreted with caution since the organism is almost constantly present in the upper respiratory tract (Higgins and Gottschalk, 2006). For species identification, a minimum of biochemical tests are necessary, as described above. When the organism is isolated from diseased animals and serotyping is available, an α-hemolytic *Streptococcus* that produces amylase but not acetoin can be considered as *S. suis* (Devriese et al., 1991). Serotype-specific isolation from contaminated tissues, such as tonsils, may also be carried out using a reported immunocapture method (Gottschalk et al., 1999b). Methods such as fluorescent in situ hybridization may be used for specific detection of *S. suis* in tissues (Boye et al., 2000). Identification, as well as partial characterization with respect to serotype and the presence of virulence markers, can rapidly be performed by PCR (Marois et al., 2004; Okwumabua et al., 2003; Rasmussen and Andresen, 1998; Smith et al., 1997; Wisselink et al., 2002a).

1.1.1. Serotyping

S. suis can be classified into thirty-five serotypes (1 to 34 and 1/2) according to the CPS antigens (Higgins and Gottschalk, 2006). However, further molecular characterization has shown that serotypes 32 and 34 actually belong to another species: *Streptococcus orisratti* (see below) (Hill et al., 2005). Some of the reference strains of these serotypes originated from diseased pigs while others were isolated from the nasal cavities of clinically healthy pigs (Higgins and Gottschalk, 2006). Reference strains of serotypes 20, 31 and 33 were isolated from other animal species while the reference strain of serotype 14 was from a case of human meningitis (Gottschalk et al., 1991b; Higgins and Gottschalk, 1990; Higgins et al., 1995). Some serotypes cross-react, indicating that some capsular antigenic determinants are shared. This is the case of serotype 1/2 cross-reaction with serotype 1 and serotype 2 antisera (Perch et al., 1983). Two-way cross-reactions between serotypes 6 and 16 and one-way cross-reaction between serotypes 2 and 22 have also been described (Gottschalk et al., 1991b). Whether the different *S. suis* serotypes can be considered normal flora of the nasal cavity or they represent actual pathogens

remains controversial (Higgins and Gottschalk, 2006). The serotype 2 is the most prevalent serotype isolated from diseased pigs in most countries and, in addition, it is the most virulent (Higgins and Gottschalk, 2006). Although serotype 2 isolates predominate in most countries, the distribution may differ depending on the geographical location. For example, the prevalence of serotype 2 strains recovered from diseased animals in Canada and the United States remains relatively low (below 25%) (Messier et al., 2008; Article V, Appendix). In some European and Asian countries, however, serotype 2 is isolated more frequently (Berthelot-Herault et al., 2000; Lun et al., 2007; Takamatsu et al., 2008c; Wisselink et al., 2000; Ye et al., 2006). Under specific circumstances, other serotypes have been found to be highly prevalent, i.e., serotype 14 in the United Kingdom (Heath et al., 1996) and serotypes 1/2 and 5 in Canada (Lapointe et al., 2002). Serotype 7 predominated for several years in Scandinavian countries but, later, serotype 2 was reported to be the most prevalent (Higgins and Gottschalk, 1990, 2006). Nevertheless, isolates associated with disease belong, for the most part, to serotypes 1 to 8 and 1/2. The number of untypable strains isolated from diseased animals (mainly recovered from cases of sporadic disease) is, in general, relatively low (Higgins and Gottschalk, 2006; Messier et al., 2008). Serotyping is an important step of the routine diagnostic procedures. Different techniques have been described, but nowadays most laboratories use the coagglutination technique (Gottschalk et al., 1993; Higgins and Gottschalk, 1990).

1.1.2. Molecular typing

Genetic differences between and within serotypes of *S. suis* have been detected with multilocus sequence typing (MLST, discussed in a separate section), multilocus enzyme electrophoresis (MLEE) (Hampson et al., 1993; Mwaniki et al., 1994), restriction endonuclease analysis (Beaudoin et al., 1992a; Mogollon et al., 1991), ribotyping (Okwumabua et al., 1995; Rasmussen et al., 1999; Rasmussen and Andresen, 1998; Smith et al., 1997; Staats et al., 1998), sequencing of the *cpn60* gene encoding a 60-kDA chaperonin (Brousseau et al., 2001), pulse field gel electrophoresis (PFGE) (Allgaier et al., 2001; Berthelot-Herault et al., 2002; Luey et al., 2007), and randomly amplified polymorphic DNA (RAPD) (Chatellier et al., 1999; Martinez et al., 2002). Recently, an approach based on PCR amplification of a fragment of rDNA sequences, including a part of the 16S and 23S genes and the 16S-23S rDNA intergenic spacer region (ISR), followed by restriction fragment length polymorphism (RFLP) analysis (ISR-RFLP) was also described (Marois et al., 2006). Although many of these studies involved small numbers of

isolates, focused on isolates representing a single serotype or geographical location, and used a variety of traditional approaches such that it is difficult to compare results among studies, a general conclusion that can be drawn from these studies is that genetic diversity among members of the *S. suis* species is important (Higgins and Gottschalk, 2006). This fact should be taken into account in diagnosis, surveillance, and control of the disease (Higgins and Gottschalk, 2006). In addition, it has been suggested that isolates from clinically healthy animals may be very heterogeneous, in contrast to most isolates from cases of diseased animals or humans (Allgaier et al., 2001; Beaudoin et al., 1992a; King et al., 2001; Staats et al., 1998). Moreover, there is considerable evidence that virulent isolates are genetically distinct from avirulent isolates, suggesting a clonal association with virulence in *S. suis*. For instance, it has been observed that most serotype 2 isolates from septicemia represented a single ribotype, whereas less-virulent isolates were genetically heterogeneous (Staats et al., 1998). Similarly, virulent isolates of serotypes 1 and 2 had distinct ribotype profiles, and particular ribotype profiles were clearly associated with clinical-pathology observations (Rasmussen et al., 1999; Smith et al., 1997). Similar results were observed using RAPD for serotypes 2 and 1/2 (Chatellier et al., 1999; Martinez et al., 2002). A recent study of ninety-nine strains by macrorestriction of DNA revealed four major clusters, one of which was strongly associated with invasive disease, and indicated that isolates from pigs with meningitis and septicemia showed a significantly higher degree of genetic homogeneity than isolates from pigs with pneumonia or isolates from healthy pigs (Allgaier et al., 2001).

An unforeseen outcome of the use of molecular typing tools was the recent reclassification of two *S. suis* serotypes into another species. Indeed, further analysis of the sequences of 16S rDNA and *cpn60* genes of the previously described thirty-five serotypes of *S. suis* led to the observation that serotypes 32 and 34 were significantly distinct from the others (Hill et al., 2005). Indeed, sequence data and biochemical profiles indicated that *S. suis* serotypes 32 and 34, isolated from pigs, were clustered with *S. orisratti*, a Voges-Proskauer negative, alpha-hemolytic, esculin-hydrolytic, species isolated from the teeth of rats which reacts with Lancefield group A antisera. Therefore, it has been proposed that these two serotypes should not longer be considered as belonging to the *S. suis* species (Hill et al., 2005).

1.1.2.1. Multilocus sequence typing (MLST)

MLST is an adaptation of the concepts of MLEE, but identifies alleles directly from the nucleotide sequences of internal fragments of housekeeping genes rather than by comparing the electrophoretic mobilities of the enzymes they encode (Maiden et al., 1998). Sequence data can be thus readily compared readily between laboratories. Indeed, MLST is fully portable and data stored in a single expanding central MLST database (http://ssuis.mlst.net/) can be interrogated electronically via the Internet, which resolves the major problem all other molecular typing methods used for *S. suis* present, i.e., comparison of the results obtained by different laboratories (King et al., 2002; Maiden et al., 1998; Turner and Feil, 2007). An MLST scheme for *S. suis*, using the *aroA* (5-enolpyruvylshikimate 3-phosphate synthase), *cpn60*, *dpr* (peroxide resistance), *gki* (glucose kinase), *mutS* (DNA mismatch repair protein), *recA* (homologous recombination factor) and *thrA* (aspartokinase) genes has been developed (King et al., 2002). Fragments of these seven housekeeping genes from each of 294 *S. suis* isolates obtained from various *S. suis* diseases and from asymptomatic carriage representing twenty-eight serotypes and nine distinct countries of origin were sequenced. Between thirty-two and forty-six alleles per locus were identified, providing the ability to distinguish $>1.6 \times 10^{11}$ sequence types (ST). However, only ninety-two ST were identified (King et al., 2002). Among these ST, eighteen contained multiple isolates. Assignment of the ST to lineages resulted in thirty-seven being identified as unique and unrelated ST, while the remaining fifty-five were assigned to ten ST complexes (King et al., 2002). In spite of the observed genetic diversity, three major clonal groups dominated the *S. suis* population: ST complexes 1, 87 and 27 (King et al., 2002). Most striking was the ST 1 complex, containing 165 isolates, within which 141 isolates were found to represent ST 1 itself. While the ST 1 complex was strongly associated with isolates from septicemia, meningitis, and arthritis, the ST 87 and ST 27 complexes were found to contain significantly higher numbers of lung isolates (King et al., 2002). Hence, it was proposed that ST 1 isolates may represent a highly successful clone which arose relatively recently and which has rapidly spread worldwide (King et al., 2002). Indeed, repeated recovery of such indistinguishable isolates from invasive disease in different countries clearly implies that ST 1 defines strains with an increased capacity to cause disease. This might reflect a variety of factors such as increased fitness, the possession of certain virulence factors or allelic variants thereof, and the selection of clones possessing particular antibiotic resistance profiles by therapeutic or prophylactic use

(King et al., 2002). These facts seemed to be confirmed by the recent analysis of whole genome data of strains belonging to ST 1 and ST 27 complexes (Chen et al., 2007; Ye et al., 2008). Interestingly, most of the *S. suis* human isolates recovered from outbreaks in China in 1998 and 2005 (Yu et al., 2006) (see below) belonged to the ST complex 1 and while some of them were ST 1, the majority was clustered in the ST 7 (Ye et al., 2006) (Figure 2). These ST 7 isolates were more toxic to peripheral blood mononuclear cells than ST 1 strains (Ye et al., 2008; Ye et al., 2006; Zheng et al., 2008) and represented a single-locus variant of ST 1 (Ye et al., 2006). These findings strongly suggest that ST 7 is another emerging, highly virulent *S. suis* clone.

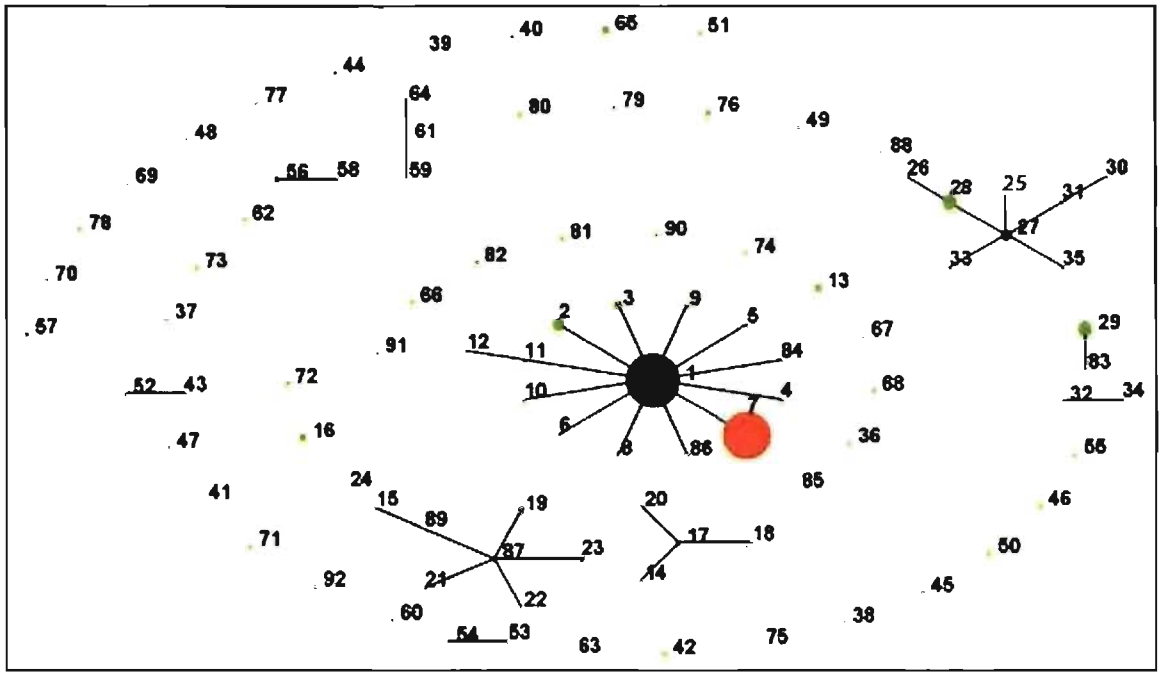

Figure 2. MLST snapshot of *S. suis*.
The entire *S. suis* multilocus sequence type database is displayed as a single eBURST diagram. The six major sequence type (ST) complexes are each denoted by a number. Primary founders (dark green) are positioned centrally in the cluster, and subgroup founders are shown in light green, except ST 7, which is shown in orange to emphasize its importance. The area of each circle in the diagram corresponds to the abundance of the isolates of the ST in the input data. Adapted from (Ye et al., 2006).

MLST has also been used to assess twenty *S. suis* Thai isolates recovered from humans during 1998–2002 (Takamatsu et al., 2008c). Serotyping showed that nineteen isolates belonged to serotype 2, while the remaining strain was serotype 14. MLST analysis resolved the twenty

isolates into eight ST. Four isolates (including the serotype 14 strain) belonged to the ST 1 complex. The remaining isolates (80 %) were assigned to the ST 27 complex. This was an interesting finding, since all the ST 27 complex isolates were isolated from the blood or cerebrospinal fluid (CSF) of the patients, suggesting a high degree of invasiveness and questioning the proposed lower virulence potential of ST 27 strains (Takamatsu et al., 2008c). Of note, before that study only four human clinical isolates had so far been reported to belong to the ST 27 complex (Takamatsu et al., 2008c). Three were isolates from Canada that belonged to ST 25 (contained in the ST 27 complex) (King et al., 2002) while the remaining one was from Japan and belonged to ST 28 (Chang et al., 2006). Apart from these human Canadian isolates, the MLST profile of North American strains has not yet been evaluated.

1.2. Infection and transmission

1.2.1. In pigs

S. suis causes meningitis, meningo-encephalitis, septicemia, arthritis and endocarditis in swine. Pericarditis, polyserositis, rhinitis, abortion and bronchopneumonia are also possible outcomes of the *S. suis* infection (Higgins and Gottschalk, 2006). The most consistent clinical signs reported for swine are neurological signs, including opisthotonus, lateral recumbency, paddling, convulsions, and ataxia. Sudden death without premonitory signs has also been reported (Reams et al., 1994). In some cases, compatible meningo-encephalitic lesions are found during the histopathological analysis but isolation of *S. suis* from the brain and meninges fails. In these cases, isolation of the pathogen from internal organs may confirm the etiology (Higgins and Gottschalk, 2006). In cases of septicemia, *S. suis* is isolated from different organs such as the spleen, liver, heart, lung, and brain. Inflammatory lesions are found in these organs on histopathological examination, but without a typical pattern of lesions (Galina et al., 1992). Suppurative or fibrinopurulent inflammation of the brain, heart, lungs and serosae are the most common histopathological observations (Higgins and Gottschalk, 2006). Infected pigs generally have clinical signs and lesions referable to either the respiratory system or to the CNS, but not both (Reams et al., 1994). *S. suis* is commonly isolated from the respiratory tract of pigs with respiratory disease. However, it is unclear whether the organism is related to pneumonia. Indeed, S. *suis* is often isolated in combination with other respiratory pathogens, such as *Pasteurella multocida, Actinobacillus pleuropneumoniae, Haemophilus parasuis, Bordetella bronchiseptica* and others (Galina et al., 1992; Reams et al., 1996), suggesting that, in these

cases, *S. suis* may act as an opportunistic pulmonary pathogen. This hypothesis is supported by the difficulty to reproduce respiratory clinical signs in experimentally infected animals. In fact pre-infection with *B. bronchiseptica* is needed for the induction of *S. suis* pneumonia during experimental trials (Vecht et al., 1992). In contrast, in pigs with meningitis or meningoencephalitis, S. *suis* is considered a primary pathogen since it is the only bacterial species isolated from the brain of these pigs (Reams et al., 1996). *S. suis* can affect pigs of all ages. However, the disease is most common following weaning and mixing, and the majority of cases occur between 3 and 12 weeks of age. The incidence of the disease varies from herd to herd and also within a herd over a period of time. No seasonal incidence has been noted in pigs (Clements et al., 1982; Clifton-Hadley, 1983). Different management practices and/or the presence of other pathogens have been suggested as predisposing factors (Dee et al., 1993). In fact, *S. suis* infection is associated with stressful conditions such as weaning, mixing and/or moving animals, and with overcrowding and poor ventilation (Dee et al., 1993). Concomitant viral infection such as Aujeszky's disease virus, porcine reproductive and respiratory syndrome (PRRS) virus and porcine circovirus (PCV) may predispose pigs to infection with *S. suis* (Feng et al., 2001; Pallares et al., 2002; Schmitt et al., 2001). The main *S. suis* reservoirs are the tonsils and nasal cavities of clinically healthy carrier pigs. *S. suis* is also commonly recovered from the lungs, the vagina, and the prepuce of pigs (Clifton-Hadley, 1984; Tarradas et al., 1994). Therefore, healthy pigs carrying pathogenic *S. suis* strains can be considered the source of infection for naive herds. The organism is both vertically and horizontally transmitted. Piglets born from sows with uterine and/or vaginal infections are either infected or become infected at birth (Cloutier et al., 2003). They also diversely and heterogeneously acquire the bacterium after birth due to close contact with the sow, her feces, and the surrounding environment (Higgins and Gottschalk, 2006). Infection of newborn piglets may also take place through the respiratory route from sow to piglets and among piglets (Berthelot-Herault et al., 2001b). It has been proposed that once a pig is infected with S. *suis* type 2, it may remain a carrier for life (Robertson and Blackmore, 1989b). Pigs may harbor different S. *suis* strains (even of different serotypes) in their nasal cavities and tonsils with no relationship with a specific pathological condition (Torremorell and Pijoan, 1998). Multiple *S. suis* serotypes from diseased animals within the same herd have also been isolated (Higgins and Gottschalk, 2006; Reams et al., 1996).

1.2.2. In other animal species

After the isolation of the serotypes 20 and 31 reference strains from diseased calves, and capsular type 33 reference strain from a diseased lamb (Gottschalk et al., 1989; Higgins et al., 1995), S. *suis* isolates of different serotypes have been recovered from ruminants, horses, cats, dogs, birds and hamsters (Devriese et al., 1992; Devriese et al., 1993; Devriese and Haesebrouck, 1992; Devriese et al., 1994; Devriese et al., 1990; Higgins and Gottschalk, 2006; Hojo et al., 2008; Hommez et al., 1988; Salasia et al., 1994). This suggests that *S. suis* may be pathogenic for more than one animal species. As described for pigs, tonsils seem to be the carriage site for *S. suis* in these animal species (Cruz Colque et al., 1993; Devriese et al., 1993; Segers et al., 1998). In addition, *S. suis* has also been found in anal swabs of dogs (Devriese et al., 1992). A possible role of these animal species as healthy carriers and as secondary reservoirs of *S. suis* should be further investigated. Finally, wild boars are a possible reservoir for *S. suis* and a major source of *S. suis* infection for hunters and poachers (Gottschalk et al., 2007; Halaby et al., 2000; Rosenkranz et al., 2003). Indeed, the prevalence of *S. suis* serotype 2 among wild boars (11%) is similar to that among domestic pigs (14%) in Germany (Baums et al., 2007).

1.2.3. In humans

S. suis infection has traditionally been considered a rare event in humans. However, it is increasingly becoming a public health concern in several Asian countries (Gottschalk et al., 2007; Segura, 2008). Indeed, in addition to the deadly outbreaks in China in 1998 and 2005 (which are described below) (Yu et al., 2006), *S. suis* has recently been described as the primary cause of adult meningitis in Vietnam as well as the third most common cause of community-acquired bacterial meningitis in Hong Kong (Hui et al., 2005; Ip et al., 2007; Mai et al., 2008) . Since the first description in Denmark in 1968 (Perch et al., 1968), human *S. suis* cases have been reported in several European (The Netherlands, Italy, Spain, United Kingdom, Belgium, Croatia, Austria, Sweden, Germany, Ireland, Hungary, France, Greece, Italy, Hungary, Portugal) and Asian countries (Japan, China, Hong Kong, Taiwan, Thailand, Singapore) as well as in Canada, the United States (US), Australia, New Zealand, and Argentina (Gottschalk et al., 2007; Taipa et al., 2008). An additional case originating in The Philippines has been reported as being a US case (Lee et al., 2008). Most cases of *S. suis* infection have been attributed to serotype 2 strains (Gottschalk et al., 2007). However, cases due to serotype 4 (Arends and Zanen, 1988), serotype

14 (Gottschalk et al., 1989), and serotype 16 (Nghia et al., 2008) have also been reported. Two human cases have been attributed to serotype 1, although the isolates from these cases were not confirmed with a serologic reaction using specific antisera (Kopic et al., 2002).

In humans, *S. suis* may produce purulent or non-purulent meningitis, endocarditis, cellulitis, peritonitis, rhabdomyolysis, arthritis, spondylodiscitis, pneumonia, uveitis and endophthalmitis (Arend et al., 1995; Huang et al., 2005; Walsh et al., 1992). An association between *S. suis* infection and colon carcinoma has also been suggested (Voutsadakis, 2006). Deafness and/or vestibular dysfunction following *S. suis* meningitis is a remarkable consequence of the infection (Lutticken et al., 1986). For unknown reasons, the recorded incidence of deafness following infection caused by *S. suis* is higher than that reported for other meningitis-causing bacteria, such as *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*, and can reach 50% and 65% in Europe and Asia, respectively (Walsh et al., 1992). Deafness, however, has never been reported in non-meningitis cases of *S. suis* human infection (Gottschalk et al., 2007). The overall case fatality rate from *S. suis* infection reaches near 13% in Europe and 20% in Asia (Huang et al., 2005). Interestingly, only three human cases of meningitis have been reported in North American (two in Canada and one in the US). These patients recovered from the infection with only minor sequelae, suggesting that North American strains of *S. suis* possess a lower virulence potential and/or that human infections caused by this organism are highly underdiagnosed in North America (Gottschalk et al., 2007).

The route of entry of the organism in humans might be small cuts in the skin, although in some cases wounds were not detected (Gottschalk et al., 2007). Additionally, oral contamination has been proposed (Bahloul et al., 2008). The bacterium may colonize the nasopharynx and the gastrointestinal tract, as suggested by diarrhea as a prodromal symptom (Fongcom et al., 2001). The incubation period ranges from a few hours to two days (Fongcom et al., 2001). Almost all cases of human infection can be ascribed to close contact with pigs or to a high degree of exposure to unprocessed pork meat, which in certain markets, has been found to be contaminated with *S. suis* (Cheung et al., 2008; Ip et al., 2007). With few exceptions, most cases (in general adult males) are pig farmers, abattoir workers, persons transporting pork, meat inspectors, butchers and veterinary practitioners (Huang et al., 2005; Tang et al., 2006; Walsh et al., 1992). Surprisingly, in a recent report from Thailand, only three out of forty-one cases had a history of occupational and behavioral exposure to pigs (Wangkaew et al., 2006). In general,

high exposure to *S. suis* may lead to a colonization of the upper respiratory tract without producing any health consequences (Gottschalk et al., 2007). Only in some cases, clinical disease may follow. Splenectomy, alcoholism and diabetes mellitus have been suggested as important predisposing factors for the development of serious *S. suis* disease (Gallagher, 2001; Huang et al., 2005; Watkins et al., 2001). Most studies showed that strains isolated from humans are phenotypically and genotypically similar to those recovered from swine within the same geographical region (Berthelot-Herault et al., 2002; Chatellier et al., 1999; Marois et al., 2006; Pedroli et al., 2003; Rehm et al., 2007; Yu et al., 2006). In addition, the virulence properties of strains isolated from pigs or humans appear to be similar (Gottschalk et al., 2007).

1.2.3.1. The 2005 human outbreak in China

An important outbreak (215 cases with a total of thirty-nine deaths) of acute disease in humans caused by *S. suis* serotype 2 was reported in the Chinese province of Sichuan in 2005 (Yu et al., 2006)., In 1998, another human outbreak had taken place in Jiangsu, China, with an outcome of fourteen deaths (Zhu et al., 2000). These outbreaks were characterized by a high incidence of systemic disease with elevated rates of mortality and a proportionally low number of cases of meningitis. In the Sichuan outbreak, human cases followed a local swine outbreak that killed more than 600 pigs (Yu et al., 2006). They were associated with backyard practices involving direct exposure to *S. suis* during the slaughtering process of pigs that had presented clinical signs of illness or had died from unknown causes (Yu et al., 2006). It is also possible that infection resulted from consumption of affected pigs. In all cases, there was no evidence of human to human infection. Chinese cases were linked at first to streptococcal toxic shock syndrome (STSS) based on the presence of the following criteria: sudden onset of high fever, diarrhea, hypotension, blood spots and petechia, clear erythematous blanching rash and dysfunction of multiple organs, such as acute respiratory distress syndrome, liver and heart failure, disseminated intravascular coagulation and acute renal failure (Tang et al., 2006; Yu et al., 2006). However, the presence of superantigens could not be demonstrated in the strains isolated during the Sichuan outbreak (Tang et al., 2006; Ye et al., 2006; Yu et al., 2006). The 2005 isolates were all *S. suis* serotype 2 (Tang et al., 2006; Ye et al., 2006; Yu et al., 2006). Using RFLP, PFGE and ribotyping, strains involved in both the human and swine outbreaks were shown to belong to the same clone responsible for the 1998 outbreak in the Jiangsu province (Tang et al.,

2006; Ye et al., 2006; Yu et al., 2006). Using MLST, all but two strains from the 2005 outbreak were classified as ST 7 (ST 1 complex) (Ye et al., 2006).

Although the Sichuan episode was the first very large human outbreak with many patients presenting severe acute symptoms, there is one previous report of an STSS caused by *S. suis* (Suankratay et al., 2004).There are many other reports in the literature indicating severe cases of *S. suis* sepsis with shock, multiple organ failure, disseminated intravascular coagulation and associated purpura fulminans and death within hours (Arends et al., 1984; Bungener and Bialek, 1989; Fongcom et al., 2001; Francois et al., 1998; Kopic et al., 2002; Pedroli et al., 2003; Strangmann et al., 2002; van Jaarsveld et al., 1990). Interestingly, ten out of forty-one cases recently described in Thailand had sepsis syndrome in the absence of primary organ infection with a clinical presentation similar to that described in the Chinese outbreak (Wangkaew et al., 2006). A serious case of septic shock caused by a non-serotype 2 strain (serotype 14) has also been described (Watkins et al., 2001). However, studies performed with peripheral blood mononuclear cells and mice showed that a representative Chinese ST 7 strain was significantly more toxic and more mitogenic than the well-characterized ST 1 European virulent strain 31533 (Ye et al., 2006; Zheng et al., 2008). The genomes of representative strains involved in the outbreak have recently been sequenced (see below) (Chen et al., 2007; Yu et al., 2006).

1.3. Virulence factors of *S. suis*

Most studies on *S. suis* virulence factors have been performed using serotype 2 strains. However, one of the problems in identifying virulence factors of *S. suis* is the lack of a clear definition of virulence for this pathogen, mainly as a result of the different parameters used to define whether a strain is virulent or avirulent (Higgins and Gottschalk, 2006). Indeed, the simple presence of some proposed virulence factors does not necessarily define the strain as virulent or not. Some strains possessing a specific virulence factor are avirulent, while other strains devoid of the same factor are still virulent. Indeed, the concept of virulence may differ between different groups, since experimental infection models for *S. suis* can be misleading. For example, different studies have designated field strains as virulent or avirulent based on: 1) the clinical condition of the animal from which the strain was isolated (clinically healthy or diseased animals); 2) on the presence of virulence-related proteins; or 3) different experimental infection models, using a) different strains of mice; or b) colostrum-deprived piglets (pre-infected or

otherwise with other microorganisms); or c) piglets of different ages from either conventional or specific-pathogen-free herds (Beaudoin et al., 1992b; Berthelot-Herault et al., 2001a; Galina et al., 1994; Vecht et al., 1997). In fact, results of experimental infections of pigs with *S. suis* may depend, among other considerations, on the immunological status of the animals, the route of infection, the size of the inoculum and the presence of the organism as normal inhabitant of the upper respiratory tract of animals prior to the experimental infection (Higgins and Gottschalk, 2006). In addition, important discrepancies exist in the literature regarding even the virulence of the same strain of *S. suis* (Gottschalk et al., 1999a; Staats et al., 1999). Despite these considerations, several factors may be considered important or putatively involved in the pathogenesis of *S. suis* infection. Table 1 summarizes the virulence factors of *S. suis* proposed so far:

Table I. Characterized and proposed *S. suis* virulence factors

Virulence factor	Putative function (Verified/Putative)	Presence in virulent strains only	Virulence of mutants in swine	Reference
CpsE/F	CPS biosynthesis: Glycosyltransferases (Verified)	No	Attenuated	(Chabot-Roy et al., 2006; Charland et al., 1998; Smith et al., 1999).
Cps2C	CPS biosynthesis: Chain length determination and export. (Putative)	No	Attenuated [a]	(Smith et al., 1999; Wilson et al., 2007)
NeuB	CPS biosynthesis: N-Acetylneuraminic acid synthetase. (Putative)	No	Attenuated [a]	(Smith et al., 2000; Wilson et al., 2007)
Suilysin	Toxin (Hemolysin) (Verified)	Variable [b]	Unaffected	(Allen et al., 2001; Gottschalk et al., 1995; Jacobs et al., 1994; Lun et al., 2003)
Muramidase-released protein (MRP)	Unknown	Variable [b]	Unaffected	(Smith et al., 1992; Smith et al., 1996)
Extracellular protein factor (EF)	Unknown	Variable [b]	Unaffected	(Smith et al., 1993; Smith et al., 1996)
Fibronectin-fibrinogen binding protein (Fbps)	Adhesin: Fibronectin binding (Verified)	No	Partly attenuated	(de Greeff et al., 2002b)

α-enolase	Adhesin: Fibronectin and plaminogen binding (Verified)	No	Not available	(Esgleas et al., 2008)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Adhesin: Plaminogen, porcine tracheal rings. Plasmin acquisition (Verified)	No	Not available	(Brassard et al., 2004; Jobin et al., 2004)
Adhesin P	Hemagglutinin (Verified)	No	Not available	(Tikkanen et al., 1996)
Pili	Adhesin (Putative)	No	Not available	(Gottschalk et al., 1990; Jacques et al., 1990; Vanier et al., 2008a; Wang et al., 2008)
Dpr	Resitance to iron-mediated toxicity (Verified)	Unknown	Not available	(Haataja et al., 2002; Niven and Ekins, 2001)
Zur	Resitance to zinc-mediated toxicity (Verified)	Unknown	Not tested	(Feng et al., 2008)
Superoxide dismutase	Resitance to toxicity (Putative)	No	Not available	(Langford et al., 1991)
Arginine deiminase system (ADS)	Resitance to acidity (Verified)	No	Not tested	(Gruening et al., 2006; Winterhoff et al., 2002)
Sortase A	Protein sorting	No	Controversial results	(Osaki et al., 2002; Vanier et al., 2008b; Wang et al., 2008)
Serum opacity-like factor (OFS)	Serum opacification	No	Attenuated	(Baums et al., 2006; Takamatsu et al., 2008)
Surface antigen one (Sao)	Unknown	No	Not available	(Li et al., 2006)
Endo-beta-N-acetylglucosaminidase D (Endo D)	Degradation of host surface oligosaccharides (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
SsnA Nuclease	Degradation of host DNA (Verified)	No	Not tested	(Fontaine et al., 2004)
Hyaluronate lyase	Degradation of hyaluronic acid (Verified)	No	Not tested	(Allen et al., 2004)
Phospholipase C	Modulation of host arachidonic acid production (Verified)	No	Not available	(Jobin et al., 2005)
Collagenase	Collagen degradation	Unknown	Partly	(Wilson et al.,

	(Putative)		attenuated [a]	2007)
*lin*0523	Restriction endonuclease (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
SalK/SalR	Two-component signal transduction	Yes [c]	Attenuated	(Li et al., 2008)
RevS	Orphan transcriptional regulator	No	Partly attenuated	(de Greeff et al., 2002a)
*tre*R gene	Transcriptional regulator (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
Gene homologous to *S. mutans* SMU_61	Transcriptional regulator (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
nadR gene	Transcriptional regulator (Putative)	Unknown	Partly attenuated [a]	(Wilson et al., 2007)
38 KDa protein	Metabolism: Putative phosphoglycerate mutase	No	Not available	(Okwumabua and Chinnapapakkagari , 2005)
Glutamate dehydrogenase (GDH)	Metabolism: Glutamate dehydrogenase	No	Not available	(Okwumabua et al., 2001)
*scr*R gene	Metabolism: Sucrose operon repressor (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
*gtf*A gene	Metabolism: Sucrose phosphorylase (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
*pur*A gene	Metabolism: Adenylosuccinate synthetase (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
*pur*D gene	Metabolism: Phosphoribosylamin e-glycine ligase (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
*scr*B gene	Metabolism: Sucrose-6-phosphate hydrolase (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
cdd gene	Metabolism: Cytidine deiminase (Putative)	Unknown	Partly attenuated [a]	(Wilson et al., 2007)
*gua*A gene	Metabolism: GMP synthase (Putative)	Unknown	Unaffected [a]	(Wilson et al., 2007)
*gua*B gene	Metabolism: Inosine monophosphate dehydrogenase (Putative)	Unknown	Unaffected [a]	(Wilson et al., 2007)
LuxS and Pfs	Quorum sensing	Unknown	Not available	(Han and Lu, 2008)

Lipoprotein signal peptidase	(Putative) Lipoprotein export (Verified)	No	Unaffected	De Greeff et al., 2003)
lpp gene	Lipoprotein (Putative)	Unknown	Partly attenuated [a]	(Wilson et al., 2007)
Gene homologous to GBS SAG0907	Lipoprotein (Putative)	Unknown	Unaffected [a]	(Wilson et al., 2007)
Permease	ABC-type multidrug transporter	Unknown	Partly attenuated [a]	(Vanier et al., 2008a)
Permease	Amino acid ABC transporter (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
*man*N gene	Mannose-specific transport PTS IID (Putative)	Unknown	Attenuated [a]	(Wilson et al., 2007)
44 kDa membrane protein	Unknown	No	Not tested	(Gottschalk et al., 1992)
Gene homologous to *S. pneumoniae spr*1018	Unknown	Unknown	Partly attenuated [a]	(Wilson et al., 2007)
*gln*H gene	Unknown	Unknown	Partly attenuated [a]	(Wilson et al., 2007)

[a] Transposon mutant
[b] Results vary depending on the geographical origin of the strains studied
[c] Present only in Chinese isolates.

1.3.1. "Classical" virulence factors

Since 1993 and for several years, the capsular polysaccharide (CPS), the hemolysin known as suilysin and two virulence-related proteins known muramidase-released protein (MRP) and extracellular protein factor (EF) were considered the most important virulence factor candidates of *S. suis* (Gottschalk and Segura, 2000). Although the role played by some of these factors in *S. suis* virulence is far from being clear, for historical reasons, in this thesis they are presented first and grouped under the title "classical virulence factors".

1.3.1.1. The capsular polysaccharide (CPS)

S. suis possesses an integral, cell associated capsule (Jacques et al., 1990). The CPS of *S. suis* serotype 2 is composed of five different sugars: galactose (Gal), glucose (Glc), N-acetyl glucosamine (GlcNAc), rhamnose (Rha) and N-acetyl neuraminic (sialic) acid (NeuNAc) (Elliott and Tai, 1978; Katsumi et al., 1996). A single *cps* locus comprising twenty-two genes has been

described whose genes putatively encode the capsular biosynthetic machinery (Smith et al., 1999a; Smith et al., 2000). An actual role in capsule biosynthesis has been so far proved for the regulator gene *cpsB*, whose inactivation impairs but does not abolish the production of a capsule by *S. suis*, and for the glycosyltranferases genes *cpsEF*, whose simultaneous inactivation results in an unencapsulated phenotype (Smith et al., 1999a). For these latter two genes, however, individual mutants have not yet been contructed. Very recently, the absolute sugar composition of the serotype 2 capsule was reported to be Gal: 3, Glc: 1; GlcNAc: 1; Rha: 1; and NeuNAc: 1 (Van Calsteren et al., 2008). The fine structure of the capsule was also determined for the serotype 2 reference strain S735 (Figure 3). Methylation analysis indicated the presence of terminal Gal, 6-linked Gal, 3, 4-linked Gal, 4-linked GlcNAc, and 3, 4-linked Rha. Sialic acid was found to be terminal. When the CPS was quantitatively desialylated by mild acid hydrolysis, the terminal Gal signal increased while the 6-linked decreased, indicating a branch ending with NeuNAc –(2-6)-Gal in the native *S. suis* CPS (Van Calsteren et al., 2008). Further studies using mass spectrometry and nuclear magnetic resonance revealed a heptasaccharide repeating unit (Figure 3) (Van Calsteren et al., 2008). Comparison with other streptococci showed that the *S. suis* CPS shares certain common structural elements with the CPS produced by *Streptococcus agalactiae* (Group B *Streptococcus*, GBS), namely the sequence NeuNAc-Gal-GlcNAc-Gal. However, no rhamnose is found in the latter species and sialic acid is 2-3-linked instead of 2-6 linked (Chaffin et al., 2005).

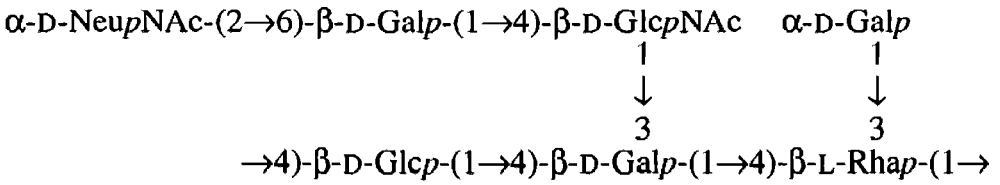

Figure 3. *S. suis* capsule structure as determined for serotype 2 reference strain S735. See the text for details. Adapted from Van Calsteren et al, 2008.

The capsule plays an important function in *S. suis* virulence. Early studies suggested that pathogenic strains were able to resist phagocytosis in porcine or human blood in the absence of anti-*S. suis* specific antibodies (Higgins and Gottschalk, 2006). These observations were confirmed with the production of isogenic unencapsulated mutants from virulent *S. suis* type 2 strains. The absence of CPS correlated with increased hydrophobicity and phagocytosis rates by

both murine and porcine macrophages (Charland et al., 1998; Smith et al., 1999a). A similar phenotype was observed for porcine neutrophils (Chabot-Roy et al., 2006). In addition, unencapsulated mutants proved to be avirulent in murine and pig models of infection (Charland et al., 1998; Smith et al., 1999a). Isolates of *S. suis* serotype 2 recovered from diseased animals have been shown to possess a thicker capsule than those isolated from clinically healthy animals (Gottschalk et al., 1991a). An increase of capsular material thickness following *in vivo* growth has been noted for virulent but not for avirulent strains (Quessy et al., 1994). On the other hand, other reports did not demonstrate any correlation between the thickness of capsular material and virulence (Higgins and Gottschalk, 2006). As discussed above, serotype 2 is considered the most virulent *S. suis* serotype. However, cells of serotype 2 reference strain are not covered by a thicker layer of CPS, compared to other serotypes (Higgins and Gottschalk, 2006). It has been suggested that the invasive ability of strains of this serotype may depend on the composition of the capsular material which contains sialic acid (Jacques et al., 1990). This latter component has already been related to virulence for other bacterial agents of meningitis (Wessels et al., 1989). However, it has been shown that virulent and avirulent strains possess a capsule of similar size with similar concentrations of sialic acid (Charland et al., 1996). In fact, resistance to clearance from the bloodstream does not rely solely on the presence of the CPS, since a well encapsulated avirulent strain is eliminated from the blood within forty-eight hours, whereas a virulent strain can persist in circulation in relatively high titers for more than five days (Higgins and Gottschalk, 2006). Actually, and despite the fact that the CPS seems to be a major virulence factor, most avirulent strains are encapsulated (Higgins and Gottschalk, 2006).

Capsular antigens are the basis for *S. suis* serotyping. Different strains within a single serotype may vary in virulence and tropism, both within and among countries (Higgins and Gottschalk, 2006). There are also differences in pathogenicity among serotypes. Several serotypes are found mainly in healthy pigs, notably serotype 21 and to a lesser extent, serotypes 17, 18, and 19 (Gottschalk et al., 1991b). The correlation between capsular antigens and virulence was the basis for the suggestion that these antigens may play a role in the pathogenesis of the disease (Robertson et al., 1991). However, this suggested correlation is not widely accepted since some strains belonging to less common serotypes have been associated with severe cases of infection (Higgins and Gottschalk, 2006).

1.3.1.2. Suilysin

Hemolysin activity is associated with virulence and pathogenicity of several bacterial species. A 54-kDa hemolysin, also known as suilysin, was identified in *S. suis* serotype 2 (Jacobs et al., 1994); subsequently a 65-kDa hemolysin from the same serotype was described (Gottschalk et al., 1995). These two proteins were shown to be the same toxin and the molecular mass variation was in fact related to purification methods (Gottschalk et al., 1995). Suilysin belongs to the family of toxins known as thiol-activated toxins or cholesterol-binding cytolytic toxins, which include streptolysin O, listeriolysin, perfringolysin, and pneumolysin (Palmer, 2001). Suilysin shares several characteristics with these toxins, such as loss of activity upon oxidation, reactivation upon reduction, inhibition by small amounts of cholesterol, formation of transmembrane pores and a multi-hit mechanism of action (Gottschalk et al., 1995). Cholesterol in the membrane of eukaryotic cells is thought to be the toxin-binding site (Alouf et al., 1991; Palmer, 2001). The gene encoding suilysin (*sly*) has been sequenced, revealing a relative high similarity with that encoding pneumolysin (Segers et al., 1998).

While several of these cytolysins have been shown to be determinants of bacterial pathogenicity, their biological roles may vary, as do the lifestyles of the bacteria producing them (Palmer, 2001). Despite the fact that a culture supernatant from a hemolysin-positive *S. suis* injected intraperitoneally failed to cause death in mice (Feder et al., 1994), a role of suilysin in virulence has been suggested since it has been shown to be cytotoxic to endothelial, epithelial and phagocytic cells (Chabot-Roy et al., 2006; Charland et al., 2000; Lalonde et al., 2000; Norton et al., 1999; Segura and Gottschalk, 2002). In addition, purified suilysin has been shown to induce the release of several pro-inflammatory cytokines by human and porcine BMEC (Vadeboncoeur et al., 2003; Vanier et al., 2004; Vanier et al., 2008b), by porcine peripheral blood cells (Segura et al., 2006) and by pig alveolar macrophages (Lun et al., 2003). Suilysin also induced the upregulation of adhesion molecules on human monocytes (Al-Numani et al., 2003). Thus, this hemolysin may play an important role in bacterial dissemination and host inflammation, which is a hallmark of *S. suis* infections. Indeed, an allelic-replacement mutant of the *sly* gene was shown to be non-toxic for murine macrophages cells (Allen et al., 2001). However, this mutant was still virulent in an intravenous pig model of infection, although total lesion scores were higher in pigs infected with the wild type (WT) strain than those infected with the mutant, suggesting that suilysin may have a role in increasing the severity of clinical signs,

and allowing bacterial colonization of infected organs to reach higher levels (Allen et al., 2001). On the other hand, an independent group also reported the production of a *sly* knockout mutant (Lun et al., 2003). *In vitro* bactericidal tests showed that both the WT and suilysin mutant were resistant to bactericidal factors present in whole pig blood. Furthermore, in a model of experimental pig infection, at either a high or a low dose of inoculation, the mutant strain induced disease similarly to the WT strain. All diseased pigs showed fever, clinical signs and developed septicemia. *S. suis* was isolated from tissue samples such as brain, submandibular lymph node, lung, spleen, liver, heart or joint (Lun et al., 2003). Thus, the results of this study confirm that suilysin is not a critical virulence factor for *S. suis* serotype 2 infections. In addition, while most Asian and European serotype 2 strains are suilysin-positive, the production of this protein has been observed in a limited number of Canadian and US serotype 2 strains (Berthelot-Herault et al., 2000; Gottschalk et al., 1998; Gottschalk et al., 2007; Okwumabua et al., 1999; Segers et al., 1998; Vecht et al., 1992; Article V, Appendix).

1.3.1.3. Muramidase-released protein (MRP) and extracellular protein (EF) factor

Two proteins, known as muramidase-released protein (MRP) and extracellular factor (EF) protein, originally associated with virulent strains, have been reported in serotype 2 strains (Vecht et al., 1991). MRP is a 136-kDa protein, present in the cell wall fraction and also released into the culture supernatant during bacterial growth, while EF is a 110-kDa protein only detected in culture supernatants (Vecht et al., 1991). Serotype 2 strains with the phenotype $MRP^{+}EF^{+}$ induced severe clinical signs of disease in pigs, but strains with the phenotype $MRP^{-}EF^{-}$ did not (Vecht et al., 1989; Vecht et al., 1992). Molecular weight variants of these two proteins were later described. Higher and lower molecular weight variants of MRP, respectively called MRP* (> 136-kDa) and MRP^{S} (< 136-kDa), and higher molecular weight EF proteins, called EF* (> 150-kDa) can be found in phenotypes such as $MRP^{*}EF^{-}$, $MRP^{S}EF^{-}$, $MRP^{S}EF^{+}$, $MRP^{-}EF^{*}$, and $MRP^{+}EF^{*}$ (Gottschalk et al., 1998; Smith et al., 1993; Wisselink et al., 2000). Strains of the last phenotype were isolated at high frequency from human patients, but caused almost no clinical signs of disease in experimentally infected pigs (Smith et al., 1993).

Although the *epf* gene encoding the EF protein has been cloned and characterized, sequence comparisons against databases did not provide information with respect to the possible functions of the EF protein (Smith et al., 1993; Smith et al., 1992). MRP, encoded by the

mrp gene, is an LPXTG protein and is therefore expected to be anchored to the cell wall peptidoglycan by sortase A (Marraffini et al., 2006). The N-terminal amino acid sequence of MRP shows some similarity with a fibronectin binding protein of *Staphylococcus aureus* (Smith et al., 1992). However, binding of MRP to human fibronectin could not be confirmed (Smith et al., 1992). So far, the function(s) of both EF and MRP proteins in the pathogenesis of the *S. suis* infection remain unclear. Isogenic mutants lacking one or both proteins appeared to be as virulent as the WT strain after experimental infection of newborn germfree piglets (Smith et al., 1996). It was suggested that the production of these proteins may only be coincidentally associated with virulence rather than being actual virulence factors. However, the association of MRP and EF with virulence is observed with strains from certain countries. For example, most North American serotype 2 strains isolated from acute cases of septicemia and/or meningitis (of either pig or human origin) are MRP and/or EF negative (Chatellier et al., 1999; Gottschalk et al., 1998) Article V, Appendix). Interestingly, as demonstrated by (RAPD) analysis, the few $MRP^{+}EF^{+}$ North American strains were clustered in the same group as European strains which shared the same phenotype (Chatellier et al., 1999). Thus, the absence of one or more of these proteins cannot necessarily be associated with a lack of virulence. Again, since the term virulence is poorly defined for *S. suis*, it is also possible that, under standardized conditions, MRP^{+} EF^{+} strains might be potentially more virulent than MRP^{-} EF^{-} strains.

1.3.2. Adhesins

Successful establishment of a bacterial infection depends on efficient adhesion of the organism to the epithelium or other cells of the host. It has been postulated that *S. suis* may use multiple adhesins to attach to host cells and that the types of adhesins expressed by a particular strain will determine its tissue specificity (Gottschalk and Segura, 2000). Expression of some adhesins may trigger internalization of the pathogen by host cells, which may enable *S. suis* to evade antibiotics and to facilitate the penetration of deeper tissues. They may also be important for the interactions of the pathogen with components of the extracellular matrix (ECM) such as fibronectin, collagen, elastin, laminin and glycosaminoglycans (GAG), like heparin, and heparan sulfate (Marastoni et al., 2008). Many of these proteins can serve as potential cell receptors for bacteria and participate in the infectious process (Nitsche-Schmitz et al., 2007). Adhesins of proteinaceous nature have been proposed to mediate binding of *S. suis* to porcine BMEC as well as to ECM proteins (Esgleas et al., 2005; Vanier et al., 2007). So far, however, only a few *bona*

fide S. suis adhesins have been identified. These adhesins are presented in this section. Readers will notice that two enzymes clearly involved in basic metabolism but showing adhesive capacities are described in this section and not in the section dealing with basic metabolism. These two proteins are α-enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

1.3.2.1. Fibronectin-fibrinogen binding protein (Ffbp)

The complete gene encoding a 64-kDa fibronectin-fibrinogen binding protein (*fbps* gene) from *S. suis* serotype 2, previously identified by a promoter-trap system as being active *in vivo* (Smith et al., 2001a), was cloned in *Escherichia coli* and sequenced (de Greeff et al., 2002b). The occurrence of the gene in various serotypes was analyzed by hybridization studies. It was shown that the *fbps* gene was present in all known serotypes of *S. suis* (except in serotypes 32 and 34, which are now considered to belong to the *S. orisratti* species, see above). The protein is expressed by strains of serotype 2. However, the expression of the 64 kDa protein by all the serotypes of *S. suis* has not been studied. Therefore, it is possible that not all serotype strains express the fibronectin-fibrinogen binding protein (de Greeff et al., 2002b). The recombinant Ffbp overexpressed in *E. coli* was able to bind human fibronectin and fibrinogen (de Greeff et al., 2002b). However, an isogenic *fbps* mutant did not present diminished binding to human fibronectin *in vitro* (Esgleas et al., 2005), suggesting either that this protein does not play a major role in fibronectin binding by *S. suis* or that redundancy for binding to this ECM protein exists. The levels of virulence of the WT and the *fbps* mutant strains were compared in a competitive infection model in young piglets (de Greeff et al., 2002b). Organ cultures showed that fibronectin-fibrinogen binding protein was not required for colonization of the tonsils but that it played a role in the colonization of the specific organs involved in an *S. suis* infection (de Greeff et al., 2002b). Therefore, the *fbps* mutant was considered partly attenuated. The induction of antibodies in piglets was verified upon infection, suggesting that this protein might be an interesting candidate for a subunit vaccine (de Greeff et al., 2002b).

1.3.2.2. Alpha-enolase

A 60-kDa immunoglobulin G (IgG)-binding protein (previously reported as a 52-kDa protein) has been purified and characterized from *S. suis* serotype 2 strains (Benkirane et al., 1997; Serhir et al., 1995). It was further observed that the *S. suis* IgG-binding protein was a

member of the heat-shock protein family Hsp60 (Benkirane et al., 1997). This protein represents a common antigen found in all the *S. suis* serotypes including both virulent and avirulent strains of *S. suis* serotype 2. The IgG-binding protein was found to be associated with the cell surface and was also released in a soluble form during bacterial growth. It reacted with a large variety of mammalian IgG, with chicken immunoglobulin Y and also with other plasma proteins (Serhir et al., 1995). Recently, it has been reported that this IgG binding protein was indeed a *S. suis* α-enolase (Esgleas et al., 2008). Gram positive α-enolases are cytoplasmic enzymes critical for sugar metabolism. However, they have been shown to be also surface located. At the surface, among other contributions to virulence, α-enolases are able to drive binding of pathogens to plasminogen (Pancholi, 2001; Pancholi and Fischetti, 1998). However, in an unprecedented finding for bacterial α-enolases, recombinant *S. suis* α-enolase expressed in *E. coli* was also able to bind fibronectin (Esgleas et al., 2008; Serhir et al., 1995). Moreover, surface plasmon resonance demonstrated that *S. suis* α-enolase binds fibronectin with high (low nanomolar) affinity (Esgleas et al., 2008). Since abolition of anchoring to the *S. suis* cell wall of LPXTG proteins does not result in full impairment of fibronectin binding by this pathogen (Vanier et al., 2008c), it might be hypothesized that α-enolase-mediated binding to fibronectin may be an important factor for *S. suis* virulence. In addition to fibronectin, and similar to previous reports in other Gram positive organisms, *S. suis* α-enolase was shown to bind plasminogen in a lysine-dependent manner (Esgleas et al., 2008). Using antisera directed against recombinant α-enolase and electron microscopy it was confirmed that the native protein is ubiquitously expressed by all *S. suis* serotypes and located at the surface of the cell (Esgleas et al., 2008) (Figure 4). Incubation of the pathogen with antibodies against α-enolase resulted in inhibition of the adhesion and invasion of porcine BMEC by *S. suis* (Esgleas et al., 2008), suggesting that this protein may contribute to the pathogenesis of *S. suis* meningitis. Interestingly, *S. suis* α-enolase has been shown to be expressed *in vivo* in pigs and highly immunogenic, as demonstrated by the use of an immunoproteomic approach (Geng et al., 2008).

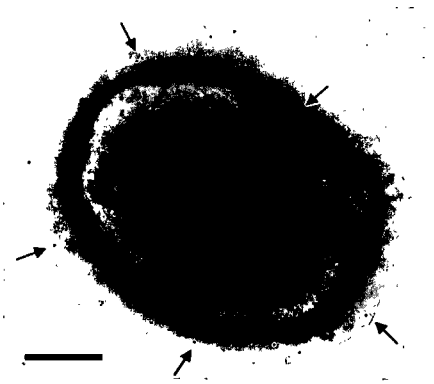

Figure 4. *S. suis* α-enolase is surface located. Immunogold electron microscopy detection of the surface location of *S. suis* α-enolase (indicated by the arrows) as revealed with rabbit anti-S. suis α-enolase IgG, followed by colloidal-gold-labelled anti-donkey IgG. Magnification: 45,000X. Adapted from Esgleas et al. 2008.

1.3.2.3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Using flow cytometry, binding of *S. suis* to albumin was observed for virulent as well as for avirulent isolates. Western blot analysis revealed that a 39-kDa *S. suis* protein was responsible, at least in part, for the binding activity (Quessy et al., 1997). Sequencing of the protein showed a high N-terminal homology with *Streptococcus pyogenes* (Group A *Streptococcus*, GAS) glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The gene encoding the GAPDH of *S. suis* was then cloned and sequenced (Brassard et al., 2004). The predicted translated 336 amino acid protein exhibited 95% sequence identity with the GAPDH from GAS and from other streptococci (Brassard et al., 2004). Recombinant *S. suis* GAPDH overexpressed in *E. coli* was found to possess functional GAPDH enzymatic activity after the purification. An adherence assay of *S. suis* to porcine tracheal rings showed a significant reduction in the adhesion of the pathogen to the rings when they were preincubated with recombinant GAPDH, suggesting the involvement of the protein in the first steps of the bacterial adhesion to host cells. In addition, at least in part, GAPDH was responsible for the binding of *S. suis* to plasminogen (Jobin et al., 2004). The *gapdh* gene has been demonstrated in isolates of serotypes 1, 9 and 7, in addition to serotype 2 (Wang and Lu, 2007). Recombinant GAPDH was used for adherence assays in another study (Wang and Lu, 2007). Data obtained demonstrated a significant reduction in adhesion of *S. suis* serotype 2 Chinese strains to HEp-2 cells pre-incubated with purified GAPDH compared to non pre-incubated controls (Wang and Lu, 2007). The contribution of GAPDH to the virulence of *S. suis* remains to be further characterized. Interestingly, it has been reported that *S. suis* highly upregulates the expression of this gene *in*

vivo in different porcine organs (Tan et al., 2008). In addition, GAPDH has been shown to be highly immunogenic in pigs (Zhang et al., 2008a).

1.3.2.4. Hemagglutinins

S. suis proteins that possess sugar-specific adherence activities for Gal, N-acetylgalactosamine (GalNAc) and sialic acid have been identified by inhibition of hemagglutination based on differences in their binding specificity (Kurl et al., 1989). Subsequently, sialylated α-2-3 poly-N-acetyl-lactosamine glycans were identified as the receptors of sialic acid-binding *S. suis* strains (Liukkonen et al., 1992) Moreover, it has been reported that galactose binding strains of *S. suis* recognize the disaccharide sequence galactosyl-α-1-4-Gal present in trihexosylceramide (Haataja et al., 1993). This ceramide is expressed on the surface of erythrocytes as well as in many pig and human tissues, and might represent the receptor for Gal binding strains in pig pharyngeal epithelium (Haataja et al., 1993). The bacterial adhesin responsible for binding to galactosyl-α-1-4-Gal has been identified (Tikkanen et al., 1995). The purified adhesin of 18 kDa was subtyped in P_O and P_N variants. The P_O variant was inhibited by Gal only, whereas P_N was inhibited by both Gal and GalNAc. The two variants have the same N-terminal amino acid sequence (Tikkanen et al., 1995). An inverse relationship between the hemagglutination activity and expression of CPS has been described, suggesting that the capsule may influence adhesin accessibility (Tikkanen et al., 1996). Interestingly, the P adhesin was present in all *S. suis* strains examined so far, was highly immunogenic and induced bactericidal activity in mice (Tikkanen et al., 1996).

1.3.2.5. Pili

Pilus-like structures were first detected in the Gram-positive species *Corynebacterium renale* (Yanagawa et al., 1968). Subsequently, pili were identified on the surface of other Gram-positive bacteria and, most recently, in all three of the principal streptococcal pathogens that cause invasive disease in humans: GAS, GBS and *S. pneumoniae* (Telford et al., 2006; Ton-That and Schneewind, 2004). Interestingly, ultrastructural studies of surface components of *S. suis* had revealed the presence of peritrichous, thin, and flexible fimbriae (Jacques et al., 1990). Morphologically similar fimbriae were observed on hemagglutinating as well as on nonhemagglutinating strains of S. *suis* (Gottschalk et al., 1990). A general feature of the Gram-

positive pili identified to date is that they comprise at least 2 protein subunits, each of which contains an LPXTG amino-acid motif (or a variant of this motif) (Proft and Baker, 2009), which is the target of dedicated sortase enzymes of the class C (Dramsi et al., 2005) that polymerize the pili. The polymerized pili are then attached to the cell wall peptidoglycan by the housekeeping sortase (Figure 5). In all Gram-positive species examined so far, the genes encoding the pilus subunits are clustered together in the same genetic locus that also contains one or more class C sortases (Proft and Baker, 2009; Scott and Zahner, 2006; Telford et al., 2006; Ton-That and Schneewind, 2004).

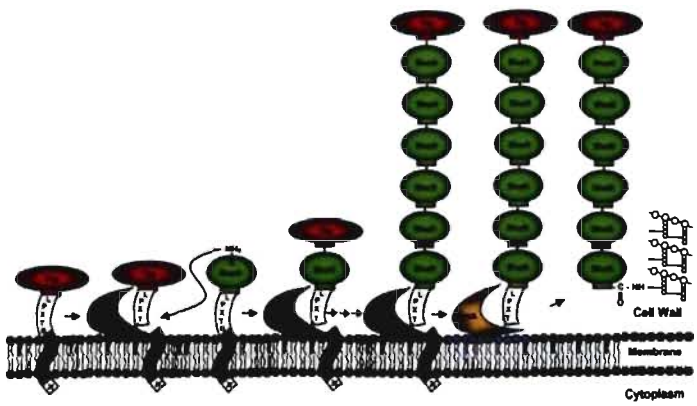

Figure 5. Simplified model for pilus assembly in Gram-positive bacteria
Following transport by the Sec system, the pilin subunits remain associated with the membrane by the hydrophobic tail of its LPXTG sorting signal. The dedicated sortase (Pip) polymerizes the growing pilus. Then, the housekeeping sortase (SrtA) covalently attaches the pilus to the peptidoglycan. Adapted from Scott and Zahner, 2006.

Pioneering studies on *S. suis* identified the housekeeping sortase SrtA as well as four class C sortases, namely SrtB, SrtC, SrtD and SrtE, (Osaki et al., 2002). More recently, the presence of clusters encoding putative pili in *S. suis* has been described (Vanier et al., 2008a; Wang et al., 2008). However, actual formation of pili and a possible role of these structures in *S. suis* virulence remain to be verified.

1.3.3. Metal uptake and resistance to toxicity

1.3.3.1. Iron

Iron is essential for the host and the pathogen, as both require this metal as a cofactor or as a prosthetic group for essential enzymes that are involved in many basic cellular functions

and metabolic pathways (Schaible and Kaufmann, 2004). Ferric iron (Fe^{3+}) is almost insoluble under aerobic, aqueous and neutral pH conditions. Specialized iron-uptake systems have been identified in most bacterial species studied so far, and they allow microorganisms to compete for this vital element within mixed microbial communities in the environment. However, so far no such systems have been reported for *S. suis*. Moreover, it has been shown that *S. suis* requires manganese, but not iron, for growth (Niven et al., 1999). Indeed, a complex medium supported normal growth of the reference strain of *S. suis* serotype 2 irrespective of the presence or absence of a high concentration (1 μM) of the iron chelating agent ethylenediamine di-o-hydroxyphenylacetic acid. Good growth was also obtained using a complex medium that had been treated with Chelex-100 to reduce the iron content, but only if this medium was supplemented with manganese (Niven et al., 1999). Growth under iron-restriction (depletion of iron in the culture medium with nitrilotriacetic acid) was also studied using a highly virulent *S. suis* strain which was grown under aerobic and/or CO_2-enriched conditions (Winterhoff et al., 2004). Decreased growth rates and down regulation of several proteins were observed, which could not be restored by addition of host iron sources such as ferritin, hemin, hemoglobin, lactoferrin or transferrin (Winterhoff et al., 2004). Accordingly, *S. suis* was not able to produce detectable amounts of siderophores. On the other hand, growth under iron-restricted conditions was fully restored by addition of Mn^{2+} (under both aerobic and CO_2-enriched conditions) or Mg^{2+} (only at CO_2-enriched conditions) (Winterhoff et al., 2004).

Although iron seems to be dispensable for *S. suis* growth, 18 unique iron-restriction-induced (*iri*) genes were identified using a promoter trap approach (Smith et al., 2001a). One of these genes was identified as the *cpsA* gene of *S. suis* which had been previously isolated as a part of the *cps* locus of *S. suis* serotype 2 and was proposed to play a role in the regulation of CPS biosynthesis (Smith et al., 1999a). A second gene, *iri-7*, showed similarity to *rpgG* of *Streptococcus mutans*, which has been shown to be required for the biosynthesis of Rha-Glc polysaccharide (Yamashita et al., 1999), suggesting a role of the *iri-7* gene in capsule biosynthesis (Smith et al., 1999a). Furthermore, it was proposed that because the size of the capsule of *S. suis* is thicker after growth *in vivo*, where free iron is scarce, upregulated expression of *cps2A* and *rpgG* under iron starvation might be expected (Smith et al., 2001a). However, a clear link between iron starvation and capsule production remains to be verified.

Other genes upregulated upon iron starvation showed similarity to ABC transporters, response regulators, housekeeping genes and proteins of unknown function (Smith et al., 2001a).

On the other hand, the readily soluble Fe^{2+} tends to react with oxygen to create reactive oxygen intermediates capable of damaging cellular macromolecules and affecting physiological processes. This is particularly important for streptococci, which are catalase negative, and therefore unable to break down the hydrogen peroxide they produce during aerobic growth. Indeed, streptococci are known to produce H_2O_2 up to mM-levels into their growth medium (Gibson et al., 2000). In addition to the endogenous production, streptococci face H_2O_2 from external sources such as activated leukocytes (Nathan and Shiloh, 2000). Toxicity of H_2O_2 is relatively weak, although it easily diffuses across biological membranes and oxidizes thiols. However, if reduced transition metal ions, especially iron, are present, H_2O_2 is nonenzymatically cleaved into highly toxic hydroxyl free radicals by Fenton chemistry ($H_2O_2 + Fe^{2+} \rightarrow .OH + OH^- + Fe^{3+}$) (Schaible and Kaufmann, 2004). *S. suis* has been shown to be able to resist damage by reactive oxygen species (Niven et al., 1999). The Dps family members constitute a distinct group of multimeric and ferritin-like iron binding proteins (up to 500 iron atoms/12-mer) that are widespread in eubacteria and archaea and implicated in oxidative stress resistance and virulence (Zhao et al., 2002). Interestingly, almost in parallel, two teams identified *S. suis* Dpr, a protein member of the Dps family which was shown to be involved in Fe^{2+} sequestration (Haataja et al., 2002; Niven and Ekins, 2001). Dpr was found to bind iron in the cytosol, an activity which proved crucial for Dpr to serve as a H_2O_2 resistance factor (Pulliainen et al., 2003). In further studies using *dpr* deletion mutants and ectopically expression of various recombinant Dpr mutant proteins, it was shown that Dpr plays a central role in *S. suis* iron homeostasis control (Haataja et al., 2002; Pulliainen et al., 2005). By analyzing the effect of single amino acid substitutions on the iron incorporation of Dpr it was shown that Fe^{2+} atoms enter the negatively charged 12-mer cavity of Dpr through four hydrophilic pores. Then, Fe^{2+} atoms are oxidized by molecular oxygen inside the 12-mer cavity at 12 identical ferroxidase centers and are subsequently stored as a Fe^{3+}-mineral (Haataja et al., 2002; Pulliainen et al., 2005).

1.3.3.2. Zinc

Similar to other transition metals such as iron, manganese, and nickel, zinc is recognized as an essential trace element for all living organisms, including a variety of bacterial pathogens.

The Zn^{2+} ion plays critical roles in various cellular processes and physiological functions by either serving as a catalytic cofactor for numerous enzymes or maintaining the structure scaffold of metal-proteins (Panina et al., 2003). However, an excess of Zn^{2+} is toxic to normal physiological processes because it can trigger the formation of hydroxyl radicals, resulting in severe damage to DNA, proteins, and lipids. Consequently, the intracellular level of Zn^{2+} must be precisely regulated to reach a dynamic balance. Bacteria have evolved complex machineries (such as efflux/influx systems) to maintain zinc homeostasis (Panina et al., 2003). The Zur (zinc uptake regulator) family members (of which the Zur protein is a representative) are involved in the precise control of Zn^{2+} homeostasis. A *zur* homologue designated as gene *310* was isolated from *S. suis* serotype 2 strain 05ZYH33, a highly invasive Chinese isolate (Feng et al., 2008). Biochemical analysis revealed that the product of gene *310* formed a dimeric protein which carried zinc ions. An isogenic gene replacement mutant of gene *310* was obtained by homologous recombination. Physiological tests demonstrated that the *310* mutant is specifically sensitive to Zn^{2+}, while functional complementation of the *310* mutant restored its resistance, suggesting that *310* is a functional member of the Zur family (Feng et al., 2008). Two-dimensional electrophoresis indicated that nine proteins in the *310* mutant are overexpressed in comparison with those in the WT strain. DNA microarray analyses suggested that 121 genes in the 310 mutant are affected, of which seventy-two are upregulated and forty-nine are downregulated. The transcriptome of the parent strain grown under a high Zn^{2+} concentration also showed 117 differentially expressed genes, with seventy-one upregulated and forty-six downregulated (Feng et al., 2008). More than 70 % of the genes differentially expressed in the 310 mutant were the same as those in the parent strain that were differentially expressed in response to high Zn^{2+} concentration, consistent with the notion that gene 310 is involved in zinc homeostasis (Feng et al., 2008). Most genes affected by *zur* deletion encode putative proteinases, of which twenty-three were upregulated and twelve downregulated (Feng et al., 2008). They included some Zn^{2+}-dependent members (e.g., NADPH, quinone reductase and lipase) involved in the basic metabolism of carbohydrates, nucleic acids, and fatty acids. There were more than 20 other genes which were influenced by *310* deletion (Feng et al., 2008), including, interestingly, *cpsC*, a gene which is putatively involved in the production of CPS (Smith et al., 1999a).

1.3.3.3. Superoxide dismutase

Superoxide ions (O_2^-) are a major antibacterial free radical in macrophage phagolysosomes. Thus, the capacity to secrete superoxide dismutases (SodA), which convert superoxide ions to molecular oxygen and H_2O_2, may be important in virulence. Indeed, these enzymes constitute one of the major defense mechanisms of cells against oxidative stress (Poyart et al., 2001; Poyart et al., 1998). The presence of a gene (*sodA*), encoding SodA as well as SOD activity was reported in *S. suis* serotype 2 strains (Langford et al., 1991). However, no correlation was observed between specific SOD activity and virulence. Therefore, it seems unlikely that the production of SodA by *S. suis* can be related to resistance of phagocytic killing by virulent isolates (Langford et al., 1991). As stated above, it has been reported that *S. suis* requires manganese, but not iron, for *in vitro* growth, and that manganese availability during growth affects the activity of the SodA enzyme (Niven et al., 1999). Further studies are needed concerning the specific role of this enzyme in *S. suis* virulence.

1.3.3.4. Arginine deiminase system (ADS)

The arginine deiminase system (ADS) catalyzes the conversion of arginine to ornithine, ammonia, and carbon dioxide. ADS are widely distributed among prokaryotic organisms and, in oral streptococci and GAS, they seem to provide protection against acidic stress by the production of ammonia (Casiano-Colon and Marquis, 1988; Degnan et al., 2000). Furthermore, it has been shown that the ADS of GAS is involved in adhesion to and invasion of epithelial cells (Degnan et al., 2000). In *S. suis* two cell wall-associated proteins with homologies to an ornithine carbamoyl-transferase (OCT) and the streptococcal acid glycoprotein (SAG) from GAS were identified (Winterhoff et al., 2002). Cloning and sequencing of the respective genes, *adiS* (80.2% homology to the *sagP* gene), and *octS* (81.2% homology to an OCT of GAS) and their adjacent upstream and downstream regions revealed that they were clustered together with two additional open reading frames (ORF). The first ORF (*orf2*) showed 59.8% homology to a gene encoding a hypothetical cytosolic protein. The second ORF (*ckS*) showed 70.1% homology to a carbamate kinase of GAS (Winterhoff et al., 2002). The genes *adiS*, *octS*, and *ckS* have been recently renamed *arcA*, *arcB*, and *arcC*, respectively (Gruening et al., 2006) (Figure 6).

Figure 6. Genetic organization of *S. suis* ADS.
The *S. suis* ADS comprises the *arcABC*, whereas genes *flpS*, *arcD*, *arcT*, *arc*h and *argR* (the last three genes are not shown in the figure) are located outside the operon. Adapted from Gruening et al, 2006.

Additional putative ADS-associated genes were identified that, however, did not belong to the *arcABC* operon. These were the *flpS* gene upstream of the *arcABC* operon with homology to the *flp* transcription regulator of *Streptococcus gordonii* and the *arcD*, *arcT*, *arcH*, and *argR* genes downstream of the *arcABC* operon with high homologies to a putative arginine-ornithine antiporter, a putative dipeptidase of *S. gordonii*, a putative β-N-acetylhexosaminidase of *S. pneumoniae*, and a putative arginine repressor of *S. gordonii*, respectively (Gruening et al., 2006). It was demonstrated that the *S. suis* ADS is inducible by arginine and reduced O_2 tension and that it is subject to carbon catabolite repression (Gruening et al., 2006). Furthermore, the comparison of an *arcA* knockout mutant, in which expression of the three operon-encoded proteins was abolished, to the WT strain showed that the *S. suis arcABC* operon contributes to survival under acidic conditions (Gruening et al., 2006).

1.3.4. Cell wall-anchored proteins

Many surface proteins which are covalently linked to the peptidoglycan of gram-positive bacteria have a consensus C-terminal motif LPXTG. In all Gram positive bacteria examined so far, this sequence is cleaved, and the processed protein is attached to an amino group of a cross-bridge in the peptidoglycan by enzymes known as housekeeping sortases (Marraffini et al., 2006). Several LPXTG proteins of *S. suis* have been involved in virulence of the organism. MRP (Smith et al., 1992) and the putative Fbps (de Greeff et al., 2002b) have been described above. For functional reasons, the LPXTG SsnA nuclease (Fontaine et al., 2004) will be discussed in a different section. Other LPXTG proteins are presented in this section, along with the housekeeping sortase SrtA

1.3.4.1. Sortase A

Using the reference strain of *S suis* serotype 2, genes encoding proteins that were homologous to sortases of other bacteria were identified (Osaki et al., 2002). One of these genes, designated *srtA*, was linked to *gyrA*, similarly to the housekeeping sortases genes of other streptococci (Igarashi, 2004; Lalioui et al., 2005; Osaki et al., 2002; Paterson and Mitchell, 2004, 2006). The deduced amino acid sequence of SrtA showed 65% identity with that of *S. gordonii*. Isogenic mutants deficient for *srtA* were generated by allelic exchange (Osaki et al., 2002). The protein fraction released from partially purified cell walls by digestion with N-acetylmuramidase was profiled by two-dimensional gel electrophoresis. More than fifteen of the protein spots were missing in the profile of the *srtA* mutant compared with that of the parent strain, and this phenotype was completely complemented by *srtA* cloned from *S. suis*. Four genes encoding proteins corresponding to such spots were identified and sequenced. The deduced translational products of the four genes possessed the LPXTG motif in their C-terminal regions (Osaki et al., 2002). The ability of the *srtA* mutant to interact with host cells and ECM proteins, as well as its virulence in a mouse infection model was further investigated. It was found that the mutant was impaired in its interactions with porcine BMEC compared to the WT strain (Vanier et al., 2008c). In addition, it showed lower level of adherence to plasma fibronectin, cellular fibronectin and collagen type I (Vanier et al., 2008c). However, disruption of *srtA* had little effect on the virulence of *S. suis* in a mouse intraperitoneal model of infection (Vanier et al., 2008c). Although these results suggest that surface proteins anchored by SrtA are required for a normal level of bacterial binding but not essential for *S. suis* virulence, different results were obtained in a parallel study carried out using an isogenic *srtA* mutant of the highly virulent Chinese strain 05ZYH33 (Wang et al., 2008). That study confirmed the role of SrtA in anchoring of cell wall proteins by using immunofluorescence analysis which revealed the absence in the cell wall of the LPXTG protein MRP. Different to the previous study, however, experimental infections of piglets showed that deletion of *srtA* attenuated the full virulence of the 05ZYH33 strain and impaired its colonizing potential in specific organs. Furthermore, the *srtA* mutant displayed significant reduction in adherence to human cells (Hep-2 and human umbilical vein endothelial cells) (Wang et al., 2008). Again, interpretation of the results is hampered by absence of a consistent definition of virulence and the use of different animal models by these two studies. However, it should be taken into account that the virulence of the

serotype 2 reference strain is controversial (Higgins and Gottschalk, 2006) and that, although the mouse reproduces several aspects of the *S. suis* infection (Dominguez-Punaro et al., 2007), the pig is the natural host of *S. suis* (Higgins and Gottschalk, 2006).

1.3.4.2. Serum opacity-like factor (OFS)

S. suis OFS presents structural homology to members of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family and a C-terminal LPXTG sorting signal (Baums et al., 2006). The N-terminal region of OFS was found to be homologous to the respective regions of fibronectin-binding protein A of *Streptococcus dysgalactiae* and the serum opacity factor of GAS. Similar to these two proteins, the N-terminal domain of OFS was able to opacify horse serum (Baums et al., 2006). Heterologous expression of *S. suis* OFS in *Lactococcus lactis* demonstrated that a high level of expression of OFS is sufficient to provide surface-associated serum opacification activity. However, the C-terminal repetitive sequence elements of OFS differed significantly from the respective repeat regions of *S. dysgalactiae* fibronectin-binding protein and GAS serum opacity factor as well as from the consensus sequence of the fibronectin-binding repeats of MSCRAMMs. Consistently, fibronectin binding by recombinant *S. suis* OFS was not detectable (Baums et al., 2006). To investigate the functions of OFS in the pathogenesis of invasive *S. suis* diseases, piglets were experimentally infected with an isogenic mutant strain in which the *ofs* gene had been inactivated by in-frame deletion. The mutant was severely attenuated in virulence but not in colonization (Baums et al., 2006). The prevalence and variations of the *ofs* gene among 108 *S. suis* isolates from diseased and healthy pigs, and human patients was further investigated. Regardless of their origins, only approximately 30 % of the isolates possessed a functional OFS (Takamatsu et al., 2008b). These results show that despite the observed attenuation of the *ofs* mutant, OFS contributes to the virulence of a limited number of *S. suis* isolates. Indeed, PCR screening and sequencing analysis showed that there are four allelic variants of *ofs* (designated types 1 to 4) (Takamatsu et al., 2008b). Type-1 and type-2 *ofs* genes encoded functional OFS, and sodium dodecyl sulfate (SDS) extracts of the isolates with type 1 and type 2 *ofs* opacified horse serum. In contrast, type-3 *ofs* contained a point mutation while type-4 *ofs* genes were disrupted by an insertion of an insertion sequence element or had undergone other genetic rearrangements. Consistently, the SDS extracts and culture supernatants of the isolates with type 3 and type 4 *ofs* did not show serum opacification activity (Takamatsu et al., 2008b).

1.3.4.3. Surface antigen one (Sao)

Surface antigen one (Sao) is a *S. suis* surface protein reacting with convalescent-phase sera from pigs clinically infected by *S. suis* serotype 2 (Li et al., 2006). Analysis of the predicted translated primary sequence showed that Sao presents a signal sequence for the Sec export machinery and an LPXTG anchor motif recognized by the housekeeping sortase SrtA. Interestingly, Sao did not share significant homology with other known Gram-positive proteins (Li et al., 2006). Electron microscopy studies using a Sao-specific antiserum confirmed the surface location of the Sao protein on *S. suis* and the SrtA-mediated anchoring of Sao to peptidoglycan (Li et al., 2006; Wang et al., 2008). Three allelic variants of the *sao* gene, namely *sao-S*, *sao-M*, and *sao-L*, based on the different lengths of the genes (approximately 1.5, 1.7, and 2.0 kb, respectively) have been described. These differences were determined to be caused by heterogeneity in the number of occurrences of a C-terminal repeat. Two of these *sao* variants (M and L) were found among serotype 2 strains only (Feng et al., 2007). Interestingly, Sao-specific antibodies reacted with cell lysates of twenty-eight out of thirty-three *S. suis* serotypes and twenty-five out of twenty-six serotype 2 isolates in immunoblots, suggesting the potential use of Sao for vaccination. Indeed, as described below in the vaccination section, immunization with Sao has been shown to provide protection to mice and pigs against lethal challenge with *S. suis* serotype 2 strains (Li et al., 2007). So far, the contribution of Sao to the virulence of *S. suis* remains unknown.

1.3.4.4. Endo-beta-N-acetylglucosaminidase D (Endo D)

In *S. pneumoniae* the LPXTG-containing, cell wall-anchored protein endo-beta-N-acetylglucosaminidase D (Endo D) cleaves the di-N-acetylchitobiose structure in asparagine-linked oligosaccharides. The enzyme, encoded by the *endoD* gene, generally acts on complex type oligosaccharides after removal of external sugars by neuraminidase, beta-galactosidase, and beta-N-acetylglucosaminidase (Muramatsu et al., 2001). These enzymes are cell surface-associated and contribute to pathogenesis of the bacteria by removing complex type oligosaccharides through their concerted actions. As an example, IgG becomes inefficient in complement-mediated cell lysis after removal of the oligosaccharides by exoglycosidases and Endo D (Koide et al., 1977). Recently, an LPXTG-containing EndoD homologue of *S. suis* was identified by signature-tagged mutagenesis (STM) (Wilson et al., 2007). Interestingly, the *endoD*

transposon mutant was found to be highly attenuated in both the murine and porcine models of infection. Future studies aimed at the characterization of this protein should shed light on the mechanisms by which EndoD contributes to the virulence of *S. suis*.

1.3.5. Cell wall-associated and secreted enzymes with degradative capacities

1.3.5.1. Proteases

Four major *S. suis* proteolytic activities (Arg-aminopeptidase, dipeptidyl peptidase (DPP) IV, chymotrypsin-like and caseinase) have been reported and partially characterized (Jobin and Grenier, 2003). These activities were produced not only by some *S. suis* type 2 strains, but also by the reference strains of serotypes 1, 1/2 and 3. The Arg-aminopeptidase of *S. suis* (55 kDa) was found to be both extracellular and cell-associated. It was suggested that the presence of this proteolytic activity would be useful for the production of ATP and other essential metabolic precursors (Jobin and Grenier, 2003). The DPP IV activity was found in the culture supernatant and on the cell surface of *S. suis* (Jobin and Grenier, 2003). This activity is also present in mammals on activated T cells (CD26) and in a soluble form in plasma where it regulates bioactive peptides such as cytokines (Mentlein, 1999). However, further studies are needed to evaluate the potential contribution of DPP IV (70 KDa) to the virulence of *S. suis*. The cell-associated caseinase activity was detected in all tested strains of *S. suis* serotype 2. The protease responsible for this caseinase activity (putatively belonging to the class of metalloproteases) showed a molecular mass of 36 kDa and may have a nutritional function (Jobin and Grenier, 2003). In addition, it has been suggested that it may participate in the maturation of bacterial protein precursors. However, further studies are needed to evaluate this hypothesis. The cell-associated chymotrypsin-like activity, belonging to the class of serine proteases, was detected in European, but not in North American, strains of serotype 2 tested (Jobin and Grenier, 2003). It has been suggested that these proteases may help *S. suis* meet nutritional requirements, to neutralize the host defense system and to contribute to tissue invasion and destruction. Further analysis should be carried out to determine their physiological and pathological functions. On the other hand, preliminary results indicate that *S. suis* serotype 2 strains do not produce IgG or immunoglobulin A proteases, or other enzymes with fibrinogen and fibronectin degradation. In contrast, a putative collagenase was recently identified by a STM study (Wilson et al., 2007). Although its activity has not been not verified, a transposon mutant of this putative collagenase was impaired in its virulence in both the murine and porcine models of infection (Wilson et al.,

2007). Further studies should be done to determine the prevalence of this putative collagenase among serotype 2 strains, as well as its actual role in collagen degradation.

1.3.5.2. SsnA Nuclease

A nuclease (SsnA) was identified in the virulent *S. suis* serotype 2 isolate SX332 and subsequently in each of the reference strains of serotypes 1 through 9 (Fontaine et al., 2004). Screening of 258 porcine clinical isolates from surface (nasal mucosa or palatine tonsil) or internal (joints, brain or other internal organs) locations revealed a significant relationship between expression of nuclease and isolation from an internal site. The gene *ssnA* (3,126 bp), was identified and analysis of the predicted translated SsnA sequence revealed a 35-amino-acid Sec signal sequence, a 22-amino acid DNA-binding domain, and a typical gram-positive LPXTG cell wall sorting motif (Fontaine et al., 2004). The protein was cloned and characterized. A requirement of Ca^{2+} and Mg^{2+} for SsnA activity was determined, and the substrate specificity was found to be for single- and double-stranded linear DNA (Fontaine et al., 2004). RT-PCR experiments revealed that *ssnA* is expressed throughout all stages of *S. suis* growth. Western blots with porcine anti-*S. suis* immune sera against a recombinant, truncated SsnA derivative (rSsnADelta) confirmed that SsnA is expressed *in vivo*. Furthermore, anti-rSsnA antibodies were sufficient to neutralize SsnA activity *in vitro*. Analyses of subcellular fractions of SX332 and insertional *ssnA* mutants on DNA-containing polyacrylamide gels and by Western blotting suggest that SsnA is cell wall located, consistently with a proposed SrtA-mediated anchoring to the cell wall (Fontaine et al., 2004). The actual contribution of this DNase to the virulence of *S. suis* remains to be verified.

1.3.5.3. Hyaluronate lyase

Hyaluronic acid is a linear GAG consisting of a polymer of the β-1-4 linked GlcNAc and glucuronic acid disaccharide. It is present in many mammalian tissues, including synovial fluid, cartilage, skin and brain, and forms a major part of the ECM. Hyaluronate lyase, which catalyzes the degradation of hyaluronic acid has been described in several pathogenic streptococcal species (Hynes and Walton, 2000). The gene *hyl* encoding a putative hyaluronate lyase from *S. suis* was inactivated in a serotype 7 strain (Allen et al., 2004). Using that mutant it was shown that hyaluronate lyase activity is required for *S. suis* to use the hyaluronic acid polymer as a

carbon source. Interestingly, hyaluronate lyase activity was not found in many *S. suis* strains isolated from diseased animals (Allen et al., 2004). Further work showed that although the gene *hyl* was present in 309 *bona fide S. suis* isolates representing diverse serotypes, geographic sources, and clinical backgrounds, the corresponding enzyme activity was detected in fewer than 30% of the isolates (King et al., 2004). In most cases, lack of enzymatic activity correlated with the presence of mutations (either sequence duplications or point mutations) within the *hyl* gene that resulted in a truncated polypeptide (King et al., 2004). The absence of hyaluronate lyase activity in a large majority of isolates from classic *S. suis* invasive disease (King et al., 2004), suggests that this protein is probably not a crucial virulence factor.

1.3.5.4. Phospholipase C

Phospholipase C hydrolyzes phosphatidylcholine into choline phosphate and diacylglycerol, a messenger that activates protein kinase C, an important metabolite of arachidonic acid production. Since arachidonic acid is the main precursor of prostaglandins, small lipids that enhance BBB vascular permeability and nitric oxide production (Wakelam, 1998), this activity may be of importance for *S. suis* to achieve tissue destruction and cell migration. A protein with phospholipase C activity has recently been identified in *S. suis* and shown to be active during the interactions of the bacterium with human BMEC (Jobin et al., 2004). However, the data suggested that phospholipase C required the previous action of suilysin. Indeed, even though a mutant lacking suilysin activity still possessed phospholipase C activity against ^{14}C-labelled phosphatidylcholine, it failed to induce the release of arachidonic acid from human BMEC. The phospholipase activity was found both on the surface of *S. suis* and in the culture supernatant (Jobin et al., 2004). Additional studies are required to better characterize the phospholipase C produced by *S. suis*.

1.3.6. Signal transduction

1.3.6.1. Response regulator RevS

The first described response regulator of *S. suis* serotype 2 was designated RevS (de Greeff et al., 2002a). Since no histidine kinase was found in the vicinity of the gene encoding this protein (*revS*), RevS was considered to be an orphan response regulator. The *revS* gene had previously been shown to be induced *in vivo* by the use of a promoter trap system (Smith et al.,

2001a). An isogenic knock-out *revS* mutant was generated and shown to be attenuated in colonization of specific organs in a competitive assay in piglets, indicating that *revS* plays a role in the pathogenesis of *S. suis* infections (de Greeff et al., 2002a). When the protein expression profiles of various fractions of the virulent parent strain 10 and the *revS* mutant strain were analyzed, the expression of virulence markers of *S. suis* such as MRP, EF and suilysin was not different between the parent and the mutant strains. However, one protein in the protoplast fraction, with unknown function was shown to be repressed by *revS* in the exponential growth phase of the mutant (de Greeff et al., 2002a).

1.3.6.2. The two-component signal transduction system SalK/SalR

The 89K island is a newly predicted pathogenicity island (PAI) which is specific to Chinese epidemic strains isolated from the two *S. suis* human outbreaks in China (Chen et al., 2007). Further bioinformatics analysis revealed a unique two-component signal transduction system located in the 89K PAI, which is orthologous to the SalK/SalR regulatory system of *Streptococcus salivarius* (Li et al., 2008). Suggesting that SalK/SalR is required for the full virulence of Chinese isolates of highly pathogenic *S. suis* serotype 2, inactivation by allelic exchange of *salK/R* eliminated the lethality of the strains in an experimental porcine model of infection (Li et al., 2008). Functional complementation of *salKR* into the isogenic mutant restored its full pathogenicity. Further characterization of the mutant by means of colonization experiments showed that the *salK/R* mutant failed to colonize susceptible tissues/organs of piglets. Moreover, bactericidal assays demonstrated that resistance of the mutant to neutrophil-mediated killing was greatly decreased. Consistent with its putative role as global regulator of virulence, expression microarray analysis exhibited a transcription profile alteration of twenty-six genes that were downregulated in the *salK/R* mutant (Li et al., 2008).

1.3.7. Proteins involved in basic metabolism

Some proposed *S. suis* virulence factors are enzyme involved in basic metabolism. Two of these enzymes (α-enolase and GAPDH) which clearly belong to this group have been presented above in the adhesin section. By the use of high-throughput technologies, other proteins involved in metabolism have been recently shown to contrinute to the S. suis virulence. In this section we present two other metabolic proteins considered to be virulence factor candidates.

1.3.7.1. Putative phosphoglycerate mutase(38 KDa protein)

A *S. suis* genomic DNA library was constructed and screened with a polyclonal antibody raised against the whole-cell proteins of *S. suis* serotype 2 (Okwumabua and Chinnapapakkagari, 2005). A clone that reacted with the antibody was characterized. Analysis revealed that the gene encoding the reactive protein was localized within a 2.0-kbp EcoRI DNA fragment. The nucleotide sequence contained an ORF that encoded a polypeptide of 445 amino acid residues with a calculated molecular mass of 46.4 kDa. However, the recombinant protein exhibited an electrophoretic mobility of approximately 38 kDa. At the time it was first reported, the amino acid level of the deduced primary sequence showed homology with sequences of unknown function from *S. pneumoniae* (89%) and *S. mutans* (86%) (Okwumabua and Chinnapapakkagari, 2005). The protein was later annotated as a putative phosphoglycerase mutase (GenBank Accession AF389083). Southern hybridization analysis revealed the presence of the gene in all the *S. suis* serotypes but 20, 26, 32, and 33. Consistently, Western-blot showed that these positive serotypes expressed the protein. Subcellular fractioning revealed that the protein was present in the surface and cell wall extracts. (Okwumabua and Chinnapapakkagari, 2005). The recombinant protein was reactive with serum from pigs experimentally infected with virulent strains of *S. suis* serotype 2, suggesting that the protein is immunogenic and may serve as an antigen of diagnostic importance for the detection of *S. suis* infections. In addition, pigs immunized with the recombinant 38-kDa protein mounted antibody responses to the protein and were completely protected against challenge with a homologous strain of serotype 2. The functional role of this protein with respect to pathogenesis remains to be determined, as does the potential protective immunity it may confer against a heterologous challenge.

1.3.7.2. Glutamate dehydrogenase (GDH)

Screening of the same *S. suis* genomic DNA library described above identified an additional 45-kDa protein. Restriction analysis showed that the gene encoding the 45-kDa protein was present on a 1.6-kb DraI region on the cloned chromosomal fragment. The nucleotide sequence contained an ORF that encoded a polypeptide of 448 amino acid residues with a calculated molecular mass of 48.8 kDa, in close agreement with the size observed on Western blots. Database searches revealed that the derived amino acid sequence was homologous to the sequence of GDH proteins isolated from various sources. Because of these

similarities, the protein was designated the GDH of *S. suis* (Okwumabua et al., 2001). Activity staining showed that the *S. suis* GDH activity is NAD(P)H dependent but, unlike the NAD(P)H-dependent GDH from various other sources, that of *S. suis* utilizes L-glutamate rather than alpha-ketoglutarate as the substrate (Okwumabua et al., 2001). Hybridization studies showed that the gene is conserved among serotype 2 strains. The native protein was found to be surface exposed by using immunogold labeling and electron microscopy. Antiserum raised against the purified recombinant protein was reactive with a protein of the same molecular size as the native protein in *S. suis* strains, suggesting expression of the gene in all of the isolates and antigenic conservation of the protein (Okwumabua et al., 2001). As for the 38 KDa protein described above, recombinant GDH was reactive with serum from pigs experimentally infected with a virulent strain of *S. suis* serotype 2, suggesting that the protein might also serve as an antigen of diagnostic importance (Okwumabua et al., 2001).

1.3.8. Proteins putatively involved in quorum sensing

Quorum sensing is a process by which bacteria communicate with diffusible chemical signaling molecules called autoinducers (AI). The autoinducer-2 signal (AI-2) mediates interspecies communication among Gram-positive and Gram-negative bacteria (Kolenbrander et al., 2002). AI-2 is derived from S-adenosylmethionine (SAM), which acts as a methyl donor to yield S-adenosylhomocysteine (SAH). The adenine base of SAH is removed by the Pfs enzyme to produce S-ribosylhomocysteine (SRH). SRH is the substrate of LuxS and is cleaved by the enzyme to generate homocysteine and 4, 5-dihydroxy-2, 3-pentanedione (DPD). The unstable DPD spontaneously converts into AI-2 (Schauder et al., 2001). Recently, it was shown that cell-free culture supernatants of *S. suis* induced luminescence of *Vibrio harveyi* BB170, indicating that AI-2-like molecules were produced by *S. suis* (Han and Lu, 2008). AI-2 activity peaked in the late exponential phase, and NaCl or Glc increased the production of AI-2. The maximum induction took place during the late exponential growth phase and sharply dropped in the stationary phase, suggesting that the activity of the molecule is density dependent in *S. suis* (Han and Lu, 2008). Homologues of *luxS* and *pfs*, encoding these two enzymes, were identified in the *S. suis* genome (Han and Lu, 2008). *S. suis luxS* was able to complement the *luxS*-negative phenotype of *E. coli* DH5-α. Q-RT-PCR showed that the level of transcription of *pfs,* but not that of *luxS* was highly correlated with the level of AI-2 production. Although the direct link between production of AI-2 and biofilm formation has recently been questioned (Hardie and Heurlier, 2008), further

studies on the production of this signaling molecule may help understand the recently reported ability of certain strains of *S. suis* to produce biofilms (Grenier et al., 2007) .

1.3.9. Lipoproteins

In Gram positive organisms several roles have been attributed to lipoproteins such as participation in antibiotic resistance, ABC transporter systems, adhesion and protein export (Sutcliffe and Harrington, 2002). The abundance of putative lipoproteins in typical bacterial genomes suggests that this class of proteins is of considerable physiological significance. Indeed, in streptococci other than *S. suis*, several lipoproteins have been shown to be involved in virulence (Brown et al., 2001). Lipoprotein signal peptidase (Lsp, encoded by the *lsp* gene) is involved in the removal of the signal peptide from diacylglyceride-modified prolipoproteins (Sankaran and Wu, 1994). While Lsp of *Bacillus subtilis* is not necessary for growth and viability, mutants disrupted in *lsp* have been shown to be attenuated in STM studies of *Staphylococcus aureus* (Mei et al., 1997). In *S. suis* the *lsp* gene was found to be part of a putative operon containing three overlapping genes (De Greeff et al., 2003). Besides *lsp*, this putative operon contained a gene encoding a protein that is predicted to belong to the LysR family of transcriptional regulators and a gene encoding a putative pseudouridine synthase. Interestingly, the LysR family transcriptional regulator had been previously identified as being *in vivo* regulated by the use of a promoter trap system (Smith et al., 2001a). To study the role of Lsp in the pathogenesis of *S. suis*, an isogenic knockout mutant of *lsp* was constructed in the *S. suis* serotype 2 strain 10 (De Greeff et al., 2003). Comparison of the lipoprotein profiles for strain 10 and the *lsp* mutant indicated that multiple radiolabelled bands accumulated in a higher molecular mass form, consistent with a failure to remove signal peptides from prolipoproteins. This observation was confirmed by Western blot analysis, which demonstrated the accumulation in the mutant strain of the prolipoprotein form of the *S. suis* pneumococcal surface adhesin A (PsaA) homologue (De Greeff et al., 2003). PsaA is a surface-exposed common 37-KDa multi-functional lipoprotein detected in all known serotypes of *S. pneumoniae*, which belongs to the ABC-type transport protein complex that transports Mn^{2+} (Rajam et al., 2008). The virulence of the *S. suis lsp* mutant was tested in an experimental infection model in piglets in a competitive co-colonization assay in piglets. The data showed that the *lsp* mutant strain was capable of colonizing both the tonsil and the organs specific for an *S. suis* infection as efficiently as the WT strain. This means that both strains are equally virulent, and that the knockout mutant of *lsp* is

not attenuated *in vivo*. Thus, this phenotype contrasts to that of other Gram positive pathogens defective in enzymes necessary for lipoprotein biosynthesis (Mei et al., 1997). This could suggest that lipoproteins do not play a role in the pathogenicity of *S. suis* but may also indicate that lipoproteins can be processed via an alternative route, independently of Lsp. Interestingly, data indicated that the *S. suis* PsaA homologue was not processed by an alternative signal peptidase (De Greeff et al., 2003). Further research is necessary to determine whether lipoproteins can be alternatively processed in *S. suis*. Interestingly, a recent STM study identified two genes encoding lipoproteins as being essential for virulence of *S. suis* in swine (Wilson et al., 2007). The first lipoprotein was homologous to Lpp of *S. mutans* and the second to GBS SAG0907. However, upon reevaluation of their virulence in pigs, mutants for these putative lipoprotein factors were found to be only partially attenuated (*lpp* mutant) or unaffected in virulence (*SAG0907* mutant) (Wilson et al., 2007).

1.3.10. Proteins with unknown function

1.3.10.1. 44 kDa membrane protein

A 44-kDa cell wall protein of unknown function has been proposed as a virulence factor as well as an important immunogen of *S. suis* serotype 2 after two avirulent mutants (M2 and M42) were produced from a highly virulent *S. suis* strain (Gottschalk et al., 1992). SDS-polyacrylamide gel electrophoresis (PAGE) of lysozyme treated cells demonstrated that the 44-kDa native protein, present in the parent strain, was absent in both mutants. Immunoblotting using rabbit whole cell homologous antisera revealed that the protein was strongly immunogenic. However, the actual contribution of this protein has not been further verified. Indeed, absence of virulence of these mutants may result from other genetic events that may have taken place during the procedure for isolation. In fact, mutant M2 was obtained after serial subcultures of the parent strain in the presence of rabbit anti-capsular type 2 serum and no longer possessed the type-specific capsular antigen (Gottschalk et al., 1992). On the other hand, mutant M42, obtained after passages of the parent strain at 42°C, remained encapsulated (Gottschalk et al., 1992) but it is not known whether factors other than the 44 kDa protein were also lost in this mutant in comparison to the WT strain. Despite the presence of antibodies against the capsule, antiserum prepared against M42 only partially protected mice against a challenge with the parent strain (Gottschalk et al., 1992).

1.3.10.2. Gain of virulence by complementation

A genomic library of the *S. suis* highly pathogenic strain (strain 10) was constructed and introduced into the less pathogenic strains S735 and 24 (Smith et al., 2001b). After infection of the library into young piglets pathogenic clones were selected. One specific transformant containing a 3-kb fragment of strain 10 appeared to be dominantly enriched in diseased pigs. Infection of piglets with the less pathogenic strains complemented with this fragment increased the virulence of these strains considerably (Smith et al., 2001b). In contrast, complementation with the corresponding fragment of a weakly pathogenic strain had only minor effects on virulence. Nucleotide sequence analysis of the selected fragment of the highly pathogenic strain revealed the presence of two potential ORFs, an accessory gene regulator B and a putative folylpolyglutamate synthetase, both of which were found to be mutated in the corresponding fragment of the less pathogenic strain (Smith et al., 2001b). These data strongly suggest that these genes are important for the virulence of *S. suis*. However, the actual contribution of each of these genes must be assessed by inactivation of the corresponding genes.

1.3.11. Global approaches for the identification of virulence factors

1.3.11.1. Whole genome sequencing

Availability of genome sequence data for *S. suis* is relatively novel and all the sequenced isolates belong to serotype 2. The whole genome sequences of three virulent strains isolated from human cases of toxic shock-like syndrome, Chinese MLST ST 7 strains 98HAH12 and 05ZYH33 and Chinese MLST ST 1 strain GZ1 have recently been published (Chen et al., 2007; Ye et al., 2008). A partial sequence for a Chinese avirulent strain has also been released (Chen et al., 2007). Sequencing of the European MLST ST 1 strain P1/7 (meningitis, pig) (King et al., 2002) is finished, but data has not yet been published. Sequencing of the Chinese MLST ST 7 strain SC84 (human) (Ye et al., 2006) and the Vietnamese MLST ST 1 strain BM407 (human) (Mai et al., 2008) are unfinished. These latter 3 genomes are available at the Sanger Institute (http://www.sanger.ac.uk/Projects/S_suis/). Finally, the Canadian MLST ST 25 strain 89-1591 (meningitis, pig) (King et al., 2002) sequencing project is at the stage of gap closing at the DOE Joint Genome Institute (http://genome.jgi-psf.org/draft_microbes/strsu/strsu.home.html). The genomes of the two Chinese virulent strains 98HAH12 and 05ZYH33 are 2,095,720 bp and 2,096,331 bp in length and have 2,191 and 2,194 predicted coding sequences (CDS), respectively

(Chen et al., 2007). These genomes have been compared to that of the European strain P1/7. A high degree of conservation was noted in the genome organizations and gene contents. All 3 genomes presented strong strand-biased gene distribution with a preference of genes to reside on the leading strand. Comparison showed a core genome of almost 1,900 common genes, while the number of strain-specific genes varied from forty-four to 101. Single nucleotide polymorphism (SNP) analysis of the homologous genes from the genomes of 05ZYH33 and 98HAH12 showed 744 synonymous substitutions (Ks) and 1,971 nonsynonymous substitutions (Ka). Similarly, 433 Ks and 1,194 Ka in both 98HAH12 and P1/7 were found. Both Ka/Ks ratios do not seem to be significantly different from each other, suggesting a similar intensity of selective pressure among the three serotype 2 strains (Chen et al., 2007). Nevertheless, the exact contribution of point mutations to the physiological activities and pathogenicity must be experimentally assessed. Several general pathogenicity-related pathways were identified at the genomic level besides those documented as virulence-associated factors, such as the above mentioned CPS, suilysin, EF, MRP and GDH. In detail, there were fifteen groups of putative two component signal transduction systems in strains 98HAH12 and 05ZYH33 while there were only thirteen in European strain P1/7. More than ten type two secretion systems were found in the genomes of strains 98HAH12, 05ZYH33 and P1/7. Interestingly, strains 98HAH12 and 05ZYH33 separately harbor four and three components from type four secretion systems, respectively, while strain P1/7 lacks them (Chen et al., 2007). Via the genome-wide display of G+C contents, six regions with abnormal G+C contents, varying in length from 15 kb to 40 kb were identified. Interestingly, co-linearity comparisons of 98HAH12 and 05ZYH33 genomes with that of P1/7 demonstrated an additional DNA fragment of 89 kb in the Chinese strains, which were named 89K PAIs. Further bioinformatics analysis showed that 89K shares an average G+C content of 36.8%, much lower than the average genomic G+C content of 41.1%. The 89K PAIs were 99% identical between 98HAH12 and 05ZYH33 and they encode sixty-seven and seventy-one potential ORFs, respectively.

A recent study has reported the sequencing of the Chinese human isolate GZ1 (Ye et al., 2008). This strain was isolated during the human outbreak of 2005 in Guizou province, China. Interestingly, this strain belonged to the MLST ST 1, similar to European virulent strains (King et al., 2002) and not ST 7, as was the case for the majority of human *S. suis* isolates associated with the outbreak (Ye et al., 2006). The genome of strain ST 1 GZ1 was reported to be a single circular

chromosome of 2,038,034 bp with a G+C content of 41.44%. The strain did not harbor any plasmids. The chromosome had 1,987 predicted CDS (Ye et al., 2008). Genome wide comparisons were carried out against the Chinese ST 7 strain 05ZYH33 described above and the ST 25 strain 89-1591. The genome sequence of ST1 GZ1 and the draft sequence of strain ST 25 89-1591 shared a common backbone sequence that was colinear. The homology was found to be punctuated by deletions and insertions of hundreds of genes, which were designated as "lost islands" (LI) or "acquired islands" (AI). Putative virulence genes present in both strains ST 25 89-1591 and ST 1 GZ 1 included those encoding GAPDH, sortases, GDH, ADS, hyaluronidase, Ffbp and OFS (Ye et al., 2008). LIs were found to be present in ST 25 strain 85-1591 but not in either ST 1 strain GZ1 or ST 7 strain 05ZYH33, whereas AIs were present in ST 1 strain GZ1 and ST 7 strain 05ZYH33 but not in ST 25 strain 85/1591. The AIs and LIs were also compared with ST 25 strain 89-1591 individual contiguous sequences that could not be linked to others. The results showed that ST 1 strain GZ1 has sixty-four LIs and that ST 7 05ZYH33 has fifty-four LIs (Ye et al., 2008). On the other hand, comparative analyses revealed that strain ST 1 GZ1 gained 132 AIs, including five PAIs containing genes encoding virulence factors such as suilysin, EF, SodA, extracellular serine protease, and prolipoprotein signal peptidase. Other AIs included one putatively conferring antibiotic resistance, five encoding determinants of cell structure, eleven for DNA/RNA processing, nine for regulation, six for DNA recombination, six for signal transduction, forty-three for metabolism, twenty-two for transport, one for immunogenic protein, and twenty-one of unknown function (Ye et al., 2008). The total size of the combined AIs was 478,262 bp. A comparison between ST 7 strains 05ZYH33 and SC84 was also carried out (Ye et al., 2008). A total of five AIs with sixty-four CDS comprising 66,829 bp were found to be unique to strain ST 7 SC84; these AIs were classified as ST 7-specific genomic islands and included 1) determinants for tetracycline resistance and a two-component signal-transduction system, 2) a second two-component signal-transduction system, 3) a recombinase, 4) an ABC–type metal-ion transporter, and 5) a second ABC transporter (Ye et al., 2008).

1.3.11.2. Suppression subtractive Hybridization (SSH)

In order to identify gene sequences unique to virulent strains, suppression subtractive hybridization (SSH) was conducted using the sequenced serotype 2 Chinese strain ST 7 HA9801 and the avirulent serotype 2 European isolate T15 (Jiang et al., 2008). The study identified thirty genomic regions that were absent in T15. Of these, fourteen were widely found among isolates

of Chinese origin. The DNA sequences of the thirty regions in HA9801 were determined and found to encode twenty-eight proteins that were homologous to proteins involved in various aspects of cellular surface structure, molecular synthesis, energy metabolism, regulation, transport systems and others of unknown function (Jiang et al., 2008). Unfortunately, the study did not test whether these regions were present in virulent strains of other geographical origins. However, most of these regions identified by SSH mapped to the 89K PAI, which is found in Chinese strains only (Chen et al., 2007).

1.3.11.3. *In vivo* induced genes identified by the use of a promoter trap-based approach.

A promoter selection system has been used for the identification of iron- and *in vivo*-regulated genes potentially involved in the pathogenesis of S. *suis* (Smith et al., 2001a). Chromosomal DNA fragments of *S. suis* were cloned into a plasmid in front of a promoterless erythromycin resistance gene. Subsequently, the library was used for the selection of bacteria in which erythromycin resistance was induced under iron-restricted conditions. Iron-regulated genes identified by the study have been described above in a separate section. In addition, erythromycin-resistant bacteria were selected after infection of piglets with the library and treatment of the piglets with erythromycin (Smith et al., 2001a). From the materials and methods it seems clear that the system used differed from the classic *in vivo* expression technology (IVET) (Merrell and Camilli, 2000; Rediers et al., 2005) in that a plasmid-based instead of an integrative promoter trap system was used. Therefore, this promoter trap study may have been unable to detect *in vitro* silent genes due to gene dose effects and, indeed, none of the selected genes were exclusively induced *in vivo* (Smith et al., 2001a). However, a number of interesting genes were selected (Ivs genes). Clones Ivs21,26,30 were shown to be identical to the *epf* gene which encodes the EF protein (Smith et al., 1993). Clone Ivs31 showed similarity to a Ffbp of *S. gordonii* and GAS (Smith et al., 2001a). Clones Ivs23, 24 were similar to *cpsY* of GBS involved in the regulation of capsule expression (Koskiniemi et al., 1998). In *S. suis*, this gene is not present in the *cps* locus (Smith et al., 1999a; Smith et al., 2000) and therefore its implication in CPS production was highly speculative (Smith et al., 2001a). Interestingly, a gene homologous to the competence gene *comE* of *B. subtilis* was identified (Smith et al., 2001a). This was an interesting finding, since natural competence for *S. suis* has not yet been described. Three other genes showed similarity to response regulators or were putatively involved in regulatory

functions: these were clones Ivs16 (similar to *Staphylococcus epidermidis altR*), Ivs20 (similar to *L. lactis aldR*) and ivs25 (similarity to the s*apR* gene of *S. mutans* encoding a response regulator of bacterial two-component signal-transduction systems) (Smith et al., 2001a). The latter gene was renamed revS in a subsequent study and shown to be an orphan response regulator (de Greeff et al., 2002a). In addition to the above, three Ivs clones showed similarity to genes encoding ABC transporters, four clones to housekeeping genes involved in metabolism, two clones showed similarity to transposon sequences and, finally, five Ivs clones were similar to sequences encoding proteins of unknown function (Smith et al., 2001a).

1.3.11.4. Signature tagged mutagenesis (STM) of *S. suis*

A STM system for *S. suis* serotype 2 reference strain was developed (Wilson et al., 2007) and used to identify genes essential for growth of the bacterium *in vivo*. Approximately 2,600 mutants were screened through both mouse and caesarian-derived, colostrum-deprived pig models. A total of twenty-two mutants were found to be attenuated in both the murine and porcine models of infection (Wilson et al., 2007). Among the genes identified there were *pur*A (Adenylosuccinate synthetase), *pur*D (Phosphoribosylamine-glycine ligase), *gtf*A (Sucrose phosphorylase), *scr*B/R (Sucrose-6-phosphate hydrolase/Sucrose operon repressor, and *gua*B/A (GMP synthase/Inosine monophosphate dehydrogenase), which are known to be involved in the virulence of a variety of Gram positive and Gram negative organisms (Baumler et al., 1994; Garsin et al., 2001; McFarland and Stocker, 1987; Yamashita et al., 1993). Two genes related to capsule synthesis, *cps*C, involved in CPS export (Smith et al., 1999a) and *neuB*, a putative sialic acid synthase (Smith et al., 2000) were also attenuated in both models (Wilson et al., 2007). Several of the identified genes have homologues that are putative transcriptional regulators in other Gram positive bacteria (Wilson et al., 2007). The remaining genes included a variety of hypothetical and conserved proteins with similarity to putative lipoproteins, proteases, nucleases and transporters. Since the STM mutants were obtained by transposition and complementation studies were not carried out, the presence of polar effects cannot be excluded. Interestingly, STM data demonstrated that a *manN* insertion mutant was attenuated in both mice and pigs (Wilson et al., 2007). The product of *manN* is the IID component of the mannose-specific phosphotransferase (PTS) system. In bacteria, the phosphoenolpyruvate (PEP):carbohydrate PTS accomplishes both the translocation and phosphorylation of its substrates (Postma et al., 1993). PTS consists of two general energy-coupling proteins, enzyme I

and HPr, as well as the carbohydrate-specific enzyme II proteins. In the mannose-specific PTS system, enzyme II of *S. salivarius* consists of $IIAB^{Man}$, IIC^{Man}, and IID^{Man} (Lortie et al., 2000; Vadeboncoeur et al., 2000). Neither IIC^{Man} nor IID^{Man} is phosphorylated, but all 3 proteins of the mannose-PTS are required for phosphorylation of mannose in these species (Postma et al., 1993). A separate study has shown that in *S. suis*, a single insertion of the transposon pGh9:ISS1 in *manN* was responsible for a hyper-hemolytic phenotype due to upregulation of suilysin expression by unknown mechanisms (Lun and Willson, 2005). Whether the observed attenuation of the STM *manN* mutant (Wilson et al., 2007) was the result of suilysin overexpression or a lack of a phosphotransferase metabolic function remains unclear and deserves further investigation. However, it has been reported that the growth of the *manN* mutant in media with mannose as the sole carbohydrate was similar to that of the WT strain, suggesting that IID^{Man} of *S. suis* is not essential for mannose transport, phosphorylation and metabolism (Lun and Willson, 2005). In addition, the mutant grew equally well as its WT parent in media with glucose, suggesting that *S. suis* may have other pathway(s) for glucose translocation and phosphorylation, or that the mannose-specific PTS is used by *S. suis* for glucose transport but the IID^{Man} does not play an essential role in this process (Lun and Willson, 2005).

1.3.11.5. Screening of a transposon Tn*917* mutant library

A transposon Tn*917* mutant library of *S. suis* serotype 2 strain P1/7 has been constructed (Slater et al., 2003). This library was screened for mutants showing impaired invasion of porcine BMEC. The screening led to the identification of nineteen poorly invasive mutants. Of them, five were selected and their virulence abilities assessed in a mouse model of infection. Two out of these five mutants were attenuated as measured by decreased colonization of organs and reduced mortality and morbidity. These two mutants were F8D, in which the transposon insertion interrupts an ABC-type multidrug transporter and B8A, in which Tn*917* interrupts an LPXTG protein member of a putative pili cluster (Vanier et al., 2008a). When tested in swine, these two attenuated mutants led to decreased bacterial loads in blood, less severe and delayed clinical signs, and lower plasma IL-6 levels than did infection with the WT strain. These results suggest that these two genes may contribute to the virulence of *S. suis* (Vanier et al., 2008a). However, as for the STM study, since complementation studies were not carried out the possibility of polar effects in downstream genes cannot be excluded.

1.4. Pathogenesis of the *S. suis* infection

For the most part, studies on the pathogenesis of *S. suis* infections have been carried out with serotype 2 strains (Higgins and Gottschalk, 2006). Despite increasing research in recent years, the current understanding of the subject is limited. As stated above, both vertical and horizontal transmission of *S. suis* have been demonstrated in pigs (Higgins and Gottschalk, 2006). Colonized animals may harbor the bacteria in their palatine and pharyngeal tonsils (Madsen et al., 2002a). These animals are clinically healthy and may not develop disease (carrier animals). On the other hand, some carriers will eventually develop bacteremia, septicemia and/or meningitis. For these events to happen, *S. suis* must first be able to disseminate from tonsils and/or other mucosal surfaces and reach the circulation (both blood and lymph).

1.4.1. Reaching the circulation

It is not known how *S. suis*, which is found in low quantities in tonsils, manages to traverse the first line of host defense to initiate disease. It has been postulated that the pathogen breaches the mucosal epithelia in the upper respiratory tract (Gottschalk and Segura, 2000) (Figure 7), but very few studies are available regarding the interactions between *S. suis* and epithelial cells. It has been reported that virulent *S. suis* strains can invade, to a certain extent, an epithelial cell line of human origin (Norton et al., 1999). However, invasion of epithelial cells by *S. suis* is controversial since failure to invade epithelial cells has been reported in another study (Lalonde et al., 2000). Moreover, other reports showed invasion of epithelial cells by (probably non virulent) unencapsulated strains, but not by well encapsulated strains (Benga et al., 2004; Valentin-Weigand, 2004). Suilysin positive strains may use cell disruption to reach the bloodstream and, in fact, it has been reported that suilysin is toxic for epithelial cells (Gottschalk and Segura, 2000). However, strains not producing this hemolytic toxin are also able to reach the circulation and disseminate (Higgins and Gottschalk, 2006).

1.4.2. Survival in blood, dissemination and sepsis

Once in circulation, *S. suis* is able to survive and multiplicate (Higgins and Gottschalk, 2006) (Figure 7). Survival of *S. suis* in blood has important immunological consequences that are discussed below and largely depends on the production of CPS. In fact, it has been widely documented that, in the absence of specific antibodies, the CPS protects *S. suis* from neutrophil

and monocyte/macrophage-mediated killing (Higgins and Gottschalk, 2006). Indeed, several different *in vitro* and *in vivo* experiments using isogenic unencapsulated strains have conclusively showed that absence of CPS correlates with highly augmented phagocytosis and/or killing of the mutant strain by phagocytic cells and rapid clearance from circulation (Chabot-Roy et al., 2006; Charland et al., 1998; Segura et al., 2004; Smith et al., 1999a). Suilysin positive strains seem to additionally benefit from the toxic effects of this hemolysin for survival in blood. Indeed, suilysin has been reported to be toxic not only for epithelial and endothelial cells but also for monocytes and neutrophils. Moreover, suilysin appears to affect complement-dependent killing by decreasing the opsonization of *S. suis* and the bactericidal capacity of neutrophils (Chabot-Roy et al., 2006; Charland et al., 2000; Gottschalk and Segura, 2000; Lalonde et al., 2000; Segura and Gottschalk, 2002; Segura et al., 2006). Upon entry of the pathogen into the bloodstream, bacterial cell wall components interact with the host immune system via pattern-recognition receptors (PRR), mainly CD14 and Toll-like receptor (TLR)-2 (Dominguez-Punaro et al., 2007; Graveline et al., 2007; Segura et al., 2002; Segura et al., 2006), and induce the production of pro-inflammatory mediators. Indeed, it has been shown that *S. suis* is able to induce the production of pro-inflammatory cytokines by *in vitro* cultured murine and human phagocytic cells (Segura and Gottschalk, 2002; Segura et al., 1999). Porcine whole blood stimulated by live *S. suis* also released high levels of tumor necrosis factor alpha (TNF)-α, interleukin (IL)-1β and IL-6 and intermediate levels of IL-8 and monocyte chemotactic protein (MCP)-1. The bacterial cell wall was the major cytokine-inducing component (Segura et al., 2006). Stimulation of human monocytes by whole encapsulated *S. suis* or purified cell wall components triggered the release of these cytokines and chemokines, which were significantly reduced by antibody-mediated neutralization of TLR-2 but not TLR-4. In addition, stimulation influenced the expression by monocytes of TLR-2 and CD14 mRNA (Graveline et al., 2007). Following exposure to *S. suis*, macrophages isolated from a TLR-2 knock-out murine strain showed a highly reduced, but not completely abrogated, IL-6 and MCP-1 production, suggesting the involvement of other TLRs in cytokine production. The CPS was also shown to modulate *S. suis* interactions with TLRs, since unencapsulated bacteria induced cytokine and chemokine production via TLR 2 dependent as well as independent pathways. However, the CPS contributed to MCP-1 production in a MyD88-independent manner (Graveline et al., 2007).

Although activation of the immune system during microbial infection is generally protective, septic shock may result as a consequence of excessive or poorly regulated immune response to the offending organism (Tsiotou et al., 2005). Such an unbalanced reaction may harm the host through a maladaptive release of endogenously generated inflammatory compounds, leading to septic shock or, in the case of highly mitogenic Chinese strains of ST 7(Zheng et al., 2008), to toxic shock-like syndrome (Gottschalk et al., 2007; Higgins and Gottschalk, 2006; Ye et al., 2008). Since most of the *in vitro* induced cytokines mediate responses associated with septic shock, a murine model of infection was developed and used to study the inflammatory response following *S. suis* infection (Dominguez-Punaro et al., 2007). Results showed that *S. suis* induces in mice a Th-1-type immune response characterized by the release of TNF-α and remarkably high levels of IL-6 production. Interestingly, high levels of these two immune mediators inversely correlate with survival time in patients with sepsis (Norrby-Teglund et al., 1995). High levels of IL-12 were also observed, which may influence NK and others cell and to produce interferon (IFN)-λ, suggesting a positive feedback regulation in the proinflammatory cytokine cascade (Dominguez-Punaro et al., 2007). IFN-γ contributes to immune control of invading pathogens but also, when its production is excessive or uncontrolled, may cause pathology leading to death (Goldmann et al., 2005). Levels of IL-1β were not as high as those observed for other Th-1 cytokines (Dominguez-Punaro et al., 2007). As for the chemokines, high levels of keratinocyte-derived chemokine (KC), MCP-1 (CCL2)and RANTES (CCL5) were detected during the first 36 h post infection in the septic phase of the infection, and their presence in plasma lasted longer than most of the proinflammatory cytokines tested (Dominguez-Punaro et al., 2007). Finally, the relatively delayed upregulation of the anti-inflammatory cytokine IL-10 might indicate a negative feedback mechanism to control the extent of the inflammatory response. Failure to regulate this response was observed in mice treated with neutralizing antibodies against IL-10, which resulted in an increased rate of septic shock (Dominguez-Punaro et al., 2008). These observations in mice may be extended to the pig. Indeed, pigs experimentally infected with virulent *S. suis* showed high levels of IL-6 and IL-8 (Vanier et al., 2008b). Finally, it has been recently reported in human patients that serum levels of TNF-α, IL-1β, IL-6, IL-8, IL-12p70, and IFN-γ were significantly higher in six patients with severe shock than in nine patients with meningitis only during the acute phase of *S. suis* infection in humans (Ye et al., 2008).

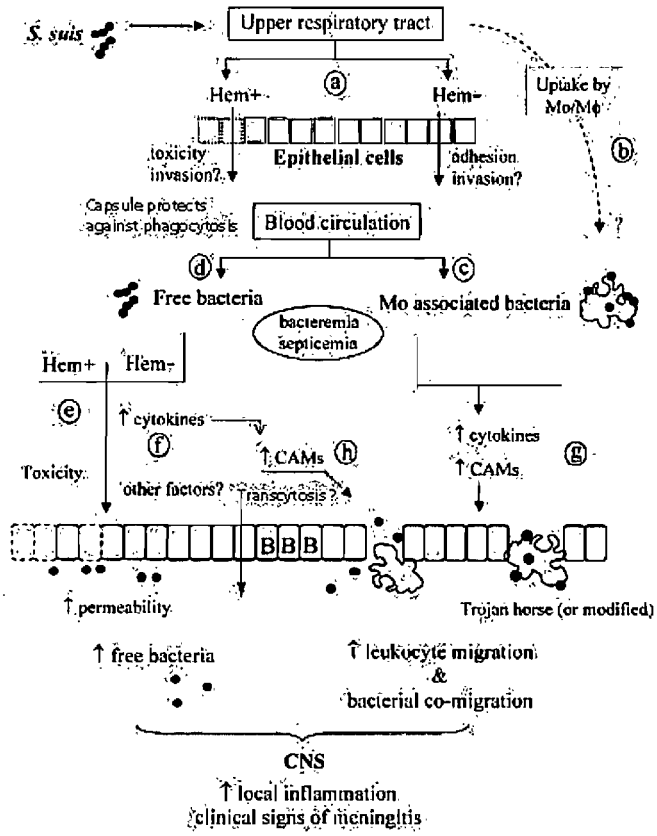

Figure 7. Current knowledge and proposed hypotheses for the different steps involved in the pathogenesis of *S. suis* infection.

Hem+: suilysin-positive strains; Hem−: suilysin-negative strains; Mo: monocytes; Mϕ: macrophages; CAMs: cell adhesion molecules; BBB: blood-brain barrier; CNS: central nervous system. Steps a and b show *S. suis* interactions with the epithelial cell layers of the upper respiratory tract and access to circulation. Hem^+ strains may use cell disruption (toxicity) and invasion to reach the bloodstream, while the mechanisms used by adhered Hem^- strains are still uncertain (Step a). *S. suis* might also be directly uptaken by Mo/Mϕ and enter to the bloodstream within circulating cells (Step b). Steps c and d describe bacterial dissemination in blood. This could be as Mo-associated (bacteria (Step c) or as free bacteria (Step d), resulting in bacteremia or septicemia. Steps e–h describe the possible mechanisms used by *S. suis* to cross the BBB. Free bacteria might enter the CNS directly by transcytosis, or by affecting BBB permeability, via cell toxicity (Hem^+ strains; Step e) or via local cytokine production (Step f). Local cytokine production might also increase CAMs expression and leukocyte migration, allowing for further bacterial trafficking (Step h). On the other hand, Mo-associated bacteria would enter the CNS via the "Trojan horse" (bacteria inside cells) or "modified" Trojan horse (bacteria adhered to cells) mechanisms favored by activated phagocyte cytokine release (Step g). In addition, *S. suis* may gain access to the CNS via disruption of the choroid plexus epithelium forming the blood-CSF barrier (not depicted). Adapted from Gottschalk and Segura, 2000.

1.4.3. *S. suis* meningitis

If death from sepsis or toxic shock-like does not occur, but the level of bacteremia remains high, then *S. suis* may cause meningitis (Gottschalk and Segura, 2000; Higgins and Gottschalk, 2006). As other blood-borne pathogens, *S. suis* must cross the BBB and/or the blood-CSF barrier in order to cause CNS infection (Figure 7). The BBB is an anatomically and

functionally unique barrier that separates the brain from the intravascular compartment and maintains the homeostasis of the CNS environment (Rubin and Staddon, 1999). The tight junctions of BMEC, which constitute microvessels, form the anatomical base of the BBB. These microvessels are covered by pericytes and outgrowths of astrocytes, which are referred to as astrocytic end-feet (Rubin and Staddon, 1999). The endothelial cells of brain microvessels differ from those in other tissues in two important aspects. Firstly, BMEC are joined by tight junctions with high electrical resistance between cells. Secondly, a relatively low number of pinocytotic vesicles, a slow rate of fluid-phase endocytosis and high densities of mitochondria are present in BMEC (Huang and Jong, 2001). The differences in both of these aspects account for the ability of the BBB to exclude certain molecules, protecting brain tissue against fluctuations in concentrations of respective components occurring in blood plasma and against destructive blood components. An early hypothesis, known as the "Trojan horse" suggested uptake of bacteria by monocytes (in the absence of specific antibodies), intracellular survival and subsequent invasion of the CNS by these intracellular *S. suis* (Williams and Blakemore, 1990). The hypothesis received support from studies carried out with flow cytometry that indicated uptake of *S. suis* by swine and human phagocytes (Busque et al., 1998). However, the number of monocytes containing bacteria in the preparations from *S. suis* bacteremic pigs was found to be very low (< 2%) (Williams and Blakemore, 1990). Indeed, a relatively high level of adhesion without phagocytosis of *S. suis* to phagocytic cells has been reported (Segura et al., 1999). Therefore, a "modified Trojan horse" hypothesis was put forward, in which the pathogen would travel and gain access to the CNS in association with monocytes (Gottschalk and Segura, 2000). If the modified Trojan horse theory (or the original Trojan horse) proved true, *S. suis* might use monocytes to reach the CNS. As the CNS is considered to be an immunoprivileged organ, normal circulation of monocytes to the CNS is controversial. However, it is accepted that the immune-privileged status of the brain is not absolute, and the permeability to some immune cells could be modified as an adaptation to the specific local microenvironment (Gottschalk and Segura, 2000).

S. suis may also travel extracellularly as free bacteria and like most meningeal pathogens such as *E. coli* K1, *Listeria monocytogenes*, *S. pneumoniae* and GBS, directly interact with cells of the BBB (Tuomanen, 1996). Interestingly, all these pathogens are able to invade BMEC (Huang and Jong, 2001). Adhesion to, but not invasion of human BMEC has been demonstrated for *S.*

suis (Charland et al., 2000). On the other hand the pathogen proved able not only to attach to but also to invade porcine BMEC (Vanier et al., 2004). Indeed, both North American and European *S. suis* serotype 2 strains adhered to and invaded these cells, as demonstrated by antibiotic protection assays and by electron microscopy. The CPS of *S. suis* partially interfered with the adhesion and invasion abilities of the bacterium, perhaps by hindering the display of putative adhesins (Vanier et al., 2004). *S. suis* survived up to 7 h within porcine BMEC (Vanier et al., 2004), which is an interesting finding since a crucial factor for the development of meningitis is the ability of pathogens to cross the BBB as live bacteria (Kim, 2006). It has been also proposed that suilysin positive strains may also disrupt the BBB through cytotoxic effects, since at high bacterial doses suilysin-positive strains were toxic for porcine BMEC. However, suilysin was not necessary for invasion, as a suilysin-negative mutant successfully invaded these cells (Vanier et al., 2004). Despite the fact that further characterization of the invasion process suggested that proteinaceous adhesins/invasins, cell wall components, lipoteichoic acid, and serum components (including fibronectin) are involved in the interactions between *S. suis* and porcine BMECs (Vanier et al., 2007), the molecular events underlying the interactions between the pathogen and these cells remain unknown.

Another CNS entry portal for *S. suis* may be the choroid plexus epithelium. Choroid plexus epithelial cells (CPEC), which also possess tight junctions, are the structural basis of the blood–CSF barrier (Strazielle and Ghersi-Egea, 2000). The choroid plexus maintains homeostasis in the CNS and also participates in neurohumoral brain modulation and neuroimmune interaction. It has been shown that CPEC are capable of restricting growth of *S. suis* upon activation with proinflammatory cytokines by creating an unfavorable microenvironment for the bacteria (Adam et al., 2004). However, and despite that finding, it has been shown that *S. suis* affects porcine CPEC barrier function and integrity by, for the most part, the induction of CPEC necrosis, although apoptosis might also play a role in the process of CPEC cell death (Tenenbaum et al., 2005; Tenenbaum et al., 2006).

Meningitis-associated brain injury and neuronal death is not mediated simply by the presence of viable bacteria but occurs as a consequence of the host reaction to bacterial components (Scheld et al., 2002). Indeed, fibrin, edema and cellular infiltrates in the meninges are typically observed during *S. suis* meningitis (Madsen et al., 2002b). *S. suis* can induce the release of arachidonic acid by BMEC, a mechanism that may facilitate the ability of bacteria to

penetrate the CNS and to modulate local inflammation (Jobin et al., 2005). In addition, bacterial CPS induces human macrophages to secrete prostaglandin E2 and matrix metalloproteinase 9, which also may be involved in disruption of the BBB (Jobin et al., 2006). Finally, other studies have shown that *S. suis* is able to induce the release of pro-inflammatory cytokines and chemokines by human and porcine BMEC and to up-regulate the expression of adhesion molecules on human monocytes with consequent increased adherence of *S. suis*-activated monocytes to endothelial cells (Al-Numani et al., 2003; Vadeboncoeur et al., 2003; Vanier et al, 2008b). *In vivo* experiments showed that infected mice that survive the septicemic phase can develop serious signs of inflammation at the CNS, with suppurative and necrotizing lesions at the meninges and brain parenchyma, especially in the cortex, hippocampus, thalamus, hypothalamus, and corpus callosum (Dominguez-Punaro et al., 2007). Bacterial antigens were detected in association with microglia residing only in the affected zones. In situ hybridization combined with immunocytochemistry showed transcriptional activation of TLR 2 and TLR 3 as well as of CD14, nuclear factor (NF)-κB, IL-1β, MCP-1 and TNF-α at these brain structures. Microglial cells were probably the main cellular sources of cytokine induction in the brain. Interestingly, early transcriptional activation of TLR 2, CD14 and inflammatory cytokines in the choroid plexus and brain endothelial cells seem to confirm that these structures are possible bacterial portals of entry into the CNS, as previously suggested (Dominguez-Punaro et al., 2007; Gottschalk and Segura, 2000; Tenenbaum et al., 2005; Tenenbaum et al., 2006; Vanier et al., 2004).

1.5. Prevention, treatment and control of the infection

1.5.1. Management practices

S. suis infection is associated with the intensification of the swine industry. Thus, control of environmental and stress factors, such as mixing and moving, using all in-all out systems of housing with adequate cleaning and drying of rooms between batches, decreasing the population density, and improving the ventilation constitute the first line of defense against the disease (Higgins and Gottschalk, 2006). These management measures coupled with strategic medication of clinically ill animals have been recommended for control and prevention of mortality caused by streptococci (Amass et al., 1996; Clifton-Hadley, 1983; Dee et al., 1993). Production technologies such as medicated early weaning and segregated early weaning have been used to improve the health status of pigs and to eliminate some infectious organisms.

However, early weaning has been shown to be ineffective for the elimination of the carrier state of *S. suis* (Dee et al., 1993; Robertson and Blackmore, 1989b; Torremorell and Pijoan, 1998). *S. suis* persists in tonsils in the presence of circulating antibodies and in pigs receiving feed medication with penicillin. This may explain how the organism can persist indefinitely in a herd and why most attempts of control or eradication by blanket medication have failed (Clifton-Hadley, 1984). It has been suggested that S. *suis* might only be eradicated by the use of extreme management procedures or by delivering all pigs by cesarean section (Dee et al., 1993).

1.5.2. Disinfectants and antibiotics

S. suis survives in feces for 104 days at 0°C, for up to 10 days at 9°C, and for up to 8 days at 22–25°C. It can also survive in dust for up to 54 days at 0°C and for up to 25 days at 9°C. However, isolation from dust stored at room temperature for 24 h failed (Clifton-Hadley, 1984). *S. suis* can also survive in water for 10 min at 60°C, making the scalding process in abattoirs a possible source of contamination. On the other hand, the organism is rapidly inactivated by disinfectants and commonly used cleansers at concentrations lower than those recommended by the manufacturers (Clifton-Hadley, 1984). Streptococci are, in general, sensitive to β-lactamins and this group of antibiotics has been used prophylactically in herds where S. *suis* serotype 2 was a problem, especially at periods of high risk, i.e., weaning (Higgins and Gottschalk, 2006). Treatment of individual cases has to be undertaken early in the course of the disease to be successful (Clifton-Hadley, 1983). Since the inflammatory reaction against infection may be detrimental in some cases, adjunctive therapy with anti-inflammatory agents is recommended for treatment of S. *suis* meningitis in pigs (Higgins and Gottschalk, 2006). Recently, it has been shown that dexamethasone prevents alteration of tight junction-associated proteins and barrier function in porcine CPEC after infection with *S. suis in vitro* (Tenenbaum et al., 2008), a fact that may help to explain the excellent results obtained when post-weaning meningitis is treated with both penicillin and dexamethasone in the very early stages of the disease (Higgins and Gottschalk, 2006). On the other hand, several cases were difficult to treat with penicillin and several moderately susceptible and some resistant strains of *S. suis* have been isolated (Gottschalk et al., 1991c; Woo and Li, 1987; Zhang et al., 2008b). It has been suggested that modifications in the penicillin-binding proteins of certain field strains, rather than β-lactamase production, are involved in the mechanism of resistance to penicillin (Higgins and Gottschalk, 2006). Thus, penicillin sensitivity can no longer be assumed for all *S.*

suis strains, and the routine treatment of S. *suis* infections with this antibiotic may need to be reevaluated if resistant strains become more prevalent (Higgins and Gottschalk, 2006). In addition to penicillin, an increase in resistance to erythromycin, clindamycin, tetracycline and fluoroquinolones has been reported (Escudero et al., 2007; Higgins and Gottschalk, 2006). *S. suis* strains recovered from humans are typically sensitive to penicillin, with a few exceptions (Gottschalk et al., 2007). Therefore, intravenous penicillin G has been used to successfully treat most cases. Since at least two cases of relapse have occurred after two and four weeks of treatment, antibiotics should be administered for a relatively long period of time (at least 6 weeks) (Gottschalk et al., 2007). As previously mentioned, hearing loss and vestibular disturbances are frequently observed sequels unrelated to antibiotic use. Resistance to norfloxacin has also been reported in strains recovered from humans (Gottschalk et al., 2007).

1.5.3. Vaccination

Passive immunization as well as maternal antibodies can protect against *S. suis* infection. Indeed, early studies have shown that transfer of serum from convalescent pigs provided pigs with complete protection against subsequent *S. suis* serotype 2 infections (Elliott et al., 1966). Protection could also be passively transferred to susceptible pigs by the inoculation of sera from pigs repeatedly immunized with live virulent *S. suis* serotype 2 strains, suggesting that humoral immunity plays an important role in *S. suis* infections (Holt et al., 1988). Maternal antibodies against *S. suis* serotype 2 can be transferred from vaccinated sows to their piglets. However, sows may respond poorly to vaccination with whole cell vaccines. Furthermore, good protection of piglets can be obtained only when titers of maternal antibodies reach a high level (Blouin et al., 1994).

Currently, no effective vaccine is available which can protect against *S. suis* infections in pigs (Higgins and Gottschalk, 2006). Available commercial vaccines are based on formalin-killed whole cell preparations that may prevent clinical disease (although the results are controversial) but do not eliminate local tissue invasion or carriership (Holt et al., 1988; 1990). Vaccination with live virulent and avirulent *S. suis* serotype 2 strains appears to confer good protection in pigs, but sometimes only after repeated immunizations (Holt et al., 1988; Busque et al., 1997; Article IV, Appendix). The efficacy of these vaccines, however, has only been evaluated after a homologous challenge with serotype 2 strains and it is not known whether whole cell vaccines

can elicit cross-protective immunity against *S. suis* (Higgins and Gottschalk, 2006). The potential of temperature-sensitive mutants of *S. suis* serotypes 1/2, 1, 2 and 3 as vaccines has been evaluated in mice (Kebede et al., 1990). All mutant strains provided protection against a challenge with a strain of homologous serotypes except for the *S. suis* serotype 1/2 mutant which provided protection to challenge with serotypes 1 and 2 strains (Kebede et al., 1990). A streptomycin-dependant mutant of *S. suis* serotype 1/2 was also tested as a vaccine. Homologous and heterologous trials in mice resulted in complete protection against challenge with *S. suis* serotypes 1 and 1/2 but only partial protection was observed against a challenge with *S. suis* serotype 2 strains (Foster et al., 1994). In many encapsulated bacteria, antibodies directed against the CPS are protective against infection (Ovodov, 2006). However, attempts to elicit an immune response in pigs by vaccination with purified *S. suis* CPS have not been successful (Elliott et al., 1980). Only when the CPS was used in combination with Freund's incomplete adjuvant, opsonizing antibodies were observed against *S. suis* serotype 2 (Elliott et al., 1980). Antibody responses against the CPS are probably not essential for full protection, since it has been reported that re-challenged pigs were completely protected against an experimental *S. suis* infection even when CPS-specific antibody levels were very low (del Campo Sepulveda et al., 1996). Moreover, heat-killed *S. suis* has been shown to elicit CPS-specific opsonizing antibodies, yet failed to provide protection against a homologous challenge with *S. suis* (Holt et al., 1990). However, comparison of the protective capacities of a live unencapsulated strain and an encapsulated bacterin indicate that the CPS (and also other bacterial components) are probably essential for full protection against homologous challenge (Wisselink et al., 2002b).

Interest has recently shifted to the experimental assessment of subunit vaccines (Higgins and Gottschalk, 2006). A subunit vaccine based on purified suilysin efficiently protected pigs against challenge with virulent *S. suis* serotype 2 strains (Segers et al., 1998). However, the absence of suilysin in a substantial number of isolates recovered from diseased pigs (Higgins and Gottschalk, 2006) limits the value of this vaccine. Attractive candidate antigens for use in a subunit vaccine are also the MRP and EF proteins since both antigens are recognized by convalescent sera of pigs infected with virulent serotype 2 strains (Vecht et al., 1991). These proteins were used for protective purposes using various different formulations. At challenge, the pigs vaccinated with MRP+EF emulsified with water in oil emulsion had high anti-MRP and

anti-EF antibody titers and were protected as effectively as pigs vaccinated with water-in-oil-formulated vaccines with bacterin (Wisselink et al., 2001). As for suilysin, however, MRP and EF proteins are absent from a large number of field strains (Higgins and Gottschalk, 2006). Recently, the Sao protein, which is highly conserved among the *S. suis* species, was shown to be recognized by convalescent swine sera (Li et al., 2006). Indeed, convalescent animals had high titers of antibodies against this protein, suggesting that Sao is a potent immunogen that is expressed during *S. suis* infection (Feng et al., 2007; Li et al., 2006). When recombinant Sao was mixed with the oil-in-water Emulsigen in a vaccination trial, it triggered a predominant production of IgG1 in piglets. However, these antibodies lacked opsonophagocytic function and did not confer protection (Li et al., 2006). This suggested that the quality of the Th1/Th2 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 Th-1-type immune response characterized by IgG2a production (Gottschalk et al., 2007). The vaccine formulation and components, such as adjuvants, can dramatically influence the vaccine-induced antibody response, including bias to type 1 or type 2 responses, which may have a significant effect on the protective efficacy of a vaccine (O'Hagan et al., 2001). Consistently, using the Th-1-directing adjuvant QuilA to promote a Th-1-type immune response, the efficacy of recombinant Sao was demonstrated by protection of mice and pigs against *S. suis* infection (Li et al., 2007). Recently, three genes of the virulent *S. suis* North American strain 89/1591, encoding putative divalent-cation-binding lipoproteins, were isolated. Recombinant proteins were produced and purified, and their immunogenic and protective abilities were analyzed (Aranda et al., 2008). All three lipoproteins (SsuiDRAFT 0103, SsuiDRAFT 0174, and SsuiDRAFT 1237) were found to be immunogenic, but only one of them (SsuiDRAFT 0103) induced a significant protective response (87.5%) against a challenge with the homologous strain. These data suggest that bacterial envelope lipoproteins putatively involved in the uptake of divalent cations other than iron may be useful for protective purposes. In recent years, a multitude of putative protective candidates for vaccination against *S. suis* infection have been reported by several research teams which have used reverse vaccinology and/or different immunoproteomics approaches (Liu et al., 2008; Okwumabua and Chinnapapakkagari, 2005; Wilson et al., 2007; Wu et al., 2008; Zhang et al., 2008a; Zhang and Lu, 2007a, b). The actual protective capacities of these candidates remain to be verified experimentally.

S. suis vaccines for humans do not exist so far (Gottschalk et al., 2007). Interestingly, there is one report of a patient with recurrent septic shock, 15 years after the first episode, due to *S. suis* serotype 2 (Francois et al., 1998). The second and fatal episode was considered as a re-infection rather than a recurrence of the previous infection. Absence of immunity after the first infection was suggested, which highlighted the importance of constant prevention in exposed workers (Francois et al., 1998). Low prevalence of antibodies against *S. suis* is also reported in healthy individuals professionally exposed to this microorganism (Elbers et al., 1999; Robertson and Blackmore, 1989a; Smith et al., 2008).

2. The selective capture of transcribed sequences (SCOTS)

Although substantial developments have recently occurred in the determination of numerous complete bacterial genome sequences, analysis of bacterial genome function by direct examination of bacterial RNA is inherently difficult due to the nature of prokaryotic mRNA. Formidable obstacles include short message half-lives, limited polyadenylation, and scarcity of material, particularly for pathogens growing in the natural environments of host cells and tissues. These barriers continue to slow progress in terms of global RNA-based analysis of *in vivo* gene expression in bacterial pathogens. SCOTS is a method for the identification of cDNAs for RNAs produced by bacterial pathogens only during growth in association with the host or under specific culture conditions (Graham and Clark-Curtiss, 1999). SCOTS directly identifies bacterial genes specifically expressed in different environments, without specialized genetic techniques, DNA microarrays, libraries, or species-specific cloning vectors, and is applicable to any microorganism from which genomic DNA can be obtained (Daigle et al., 2002; Graham and Clark-Curtiss, 1999). Unlike other recently described methods for analysis of *in vivo* gene expression (i.e. IVET, STM), SCOTS identifies potentially important genes, rather than promoter regions, and is not confounded by polar effects when genes are arranged in prokaryotic polycistronic operons. SCOTS also has the distinct advantage of being able to determine differential expression from very small numbers of bacterial cells (Daigle et al., 2002).

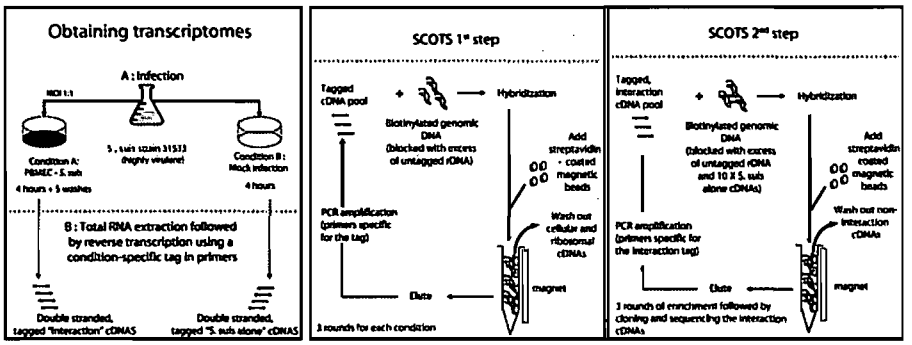

Figure 8. Schematic representation of the SCOTS technique as used in this research project.

The left panel describes the procedures for obtaining the transcriptomes. The center panel describes the enrichment in bacterial sequences representing mRNA sequences. The right panel describes the enrichment in condition-specific sequences (interaction with porcine BMEC in the example) which are then cloned and sequenced (not shown). Adapted from Graham and Clark-Curtiss, 1999.

SCOTS uses a hybridization scheme in which total RNA from a given condition (i.e., total RNA isolated from microbe-host interactions either *in vivo* or *in vitro*) is converted en masse to uniquely tagged cDNAs by using an oligonucleotide primer containing a random nonamer at their 3' end and a defined terminal sequence (tag) in the 5' end (Daigle et al., 2002; Graham and Clark-Curtiss, 1999). In parallel, total RNA from a second condition (i.e., the microbe growing in normal laboratory medium) is reverse transcribed using the same procedure but with a different tag in the primer. Then, for each condition, the bacterial cDNAs are selectively recovered from the mixture by hybridization against biotinylized genomic DNA that has been previously blocked with an excess of bacterial rDNA sequences. The recovered cDNAs are then PCR amplified using primers annealing to the specific tags and the procedure is repeated 3 times. Then, enrichment in sequences specific for one condition can be carried out by a variation of the procedure above, in which the genomic DNA is blocked with rDNA and cDNAs from the second condition. As a result, bacterial genes that are differentially expressed in the tested condition (in our example, interaction with the host) are identified (Daigle et al., 2002; Graham and Clark-Curtiss, 1999). These genes are cloned and sequenced, and differentially expressed gene sequences are compared to databases. Figure 8 shows the SCOTS procedure as used in this research project.

SCOTS has been used with success with many Gram positive and Gram negative pathogens after its initial description for elucidation of *M. tuberculosis* gene expression upon phagocytosis by human macrophages (Graham and Clark-Curtiss, 1999). Indeed, in addition to this species, SCOTS has been used to identify genes expressed *in vivo* or *in vitro* under specific conditions by animal and human pathogens such as *Salmonella* sp (Daigle et al., 2001; Faucher et al., 2005; Faucher et al., 2006), *L. monocytogenes* (Liu et al., 2002), *Helicobacter pylori* (Graham et al., 2002), *Mycobacterium avium* (Hou et al., 2002; Zhu et al., 2008), avian pathogenic *E. coli* (Dozois et al., 2003), *Actinobacillus pleuropneumoniae* (Baltes et al., 2007; Baltes and Gerlach, 2004), *Haemophilus ducreyi* (Bauer et al., 2008), and *Haemophilus parasuis* (Jin et al., 2008). In addition to the use of SCOTS as the main approach for elucidation of gene expression, the cDNAs obtained by SCOTS can be used in conjunction with microarrays to determine global gene expression by a pathogen during infection as recently demonstrated with *S. enterica* serovar Typhi (Faucher et al., 2006).

3. The Gram positive cell envelope

The two major classes of bacteria, Gram positive and Gram negative, are differentiated primarily because of differences in their surfaces. Gram positive bacteria have much thicker peptidoglycan cell wall layers and no outer membrane external to this structure. Thus, Gram-positive bacteria also lack a morphologically distinct periplasmic space, which is usually defined as the region between the cytoplasmic membrane and the outer membrane. For Gram positive bacteria, a single cytoplasmic membrane surrounded by a thick layer of peptidoglycan provides both a physical barrier for protection from the environment and a scaffold for the attachment of secondary cell wall glycopolymers (CWG) such as the wall teichoic acids (WTA) (Neuhaus and Baddiley, 2003; Weidenmaier and Peschel, 2008) as well as certain surface proteins (Dramsi et al., 2008; Marraffini et al., 2006; Navarre and Schneewind, 1999). In addition, most Gram-positive bacteria possess other surface structures. These are highly variable and include capsules, S-layer proteins, and mycolic acids that can form an outer-membrane-like structure or combinations of these elements. One constant motif of Gram-positive cell envelopes, however, is the presence of additional glycopolymers, which form part of the fabric of the cell wall and are attached either to the peptidoglycan or to membrane lipids. For the needs of this thesis, certain characteristics of peptidoglycan and selected CWG will be described.

3.1. Peptidoglycan

Peptidoglycan (murein) is an essential and specific component of the bacterial cell wall found on the outside of the cytoplasmic membrane of almost all bacteria. Its main function is to preserve cell integrity by withstanding the turgor. Any inhibition of peptidoglycan biosynthesis (mutation, antibiotic) or its specific degradation (e.g. by lysozyme) during cell growth will result in cell lysis. Peptidoglycan is intimately involved in the processes of cell growth and cell division. It also contributes to the maintenance of a defined cell shape. The *S. suis* peptidoglycan structure and traits have, to the best of our knowledge, never been described. Therefore the next six subsections describe features observed in, and conclusions drawn from, other species.

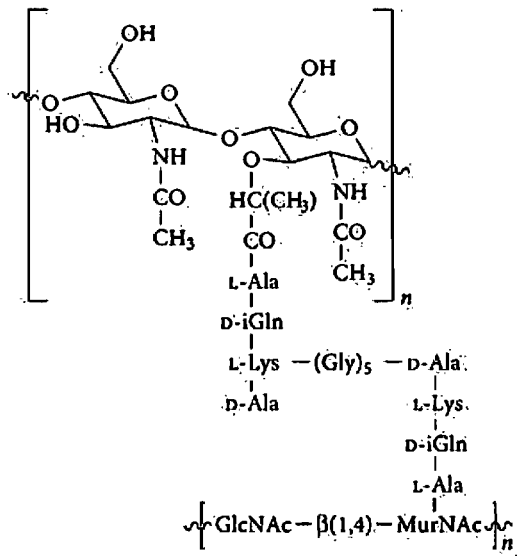

Figure 9. Peptidoglycan structure.

Peptidoglycan is a polymer of β(1-4)-linked GlcNAc and MurNAc, with all lactyl groups of MurNAc substituted with stem peptides, typically containing four alternating L- and D-amino acids. The stem peptides from adjacent strands are often crosslinked, either directly or through short peptides. The structure of the polysaccharide backbone of peptidoglycan is conserved in all bacteria. By contrast, the composition of the peptide varies: Most Gram positive cocci have L-lysine as the third amino acid (Lys-type peptidoglycan; see figure). Lys-type peptides are usually crosslinked through an interpeptide bridge that varies in length and amino-acid composition in different bacteria. The stem peptides from adjacent strands are often crosslinked, either directly or through short peptides. Adapted from Vollmer et al, 2008a.

3.1.1. General structure and characteristic features

The main structural features of peptidoglycan are linear glycan strands cross-linked by short peptides (Vollmer et al., 2008a) (Figure 9). The glycan strands are made up of alternating GlcNAc and N-acetylmuramic acid (MurNac) residues linked by β-1→4 bonds. The D-lactoyl group of each MurNac residue is substituted by a peptide stem whose composition is most often L-Ala-γ-D-Glu-*meso*-A_2pm (or L-Lys)-D-Ala-D-Ala (A_2pm, 2,6-diaminopimelic acid) in nascent

peptidoglycan, the last D-Ala residue being lost in the mature macromolecule (Vollmer et al., 2008a). Cross-linking of the glycan strands generally occurs between the carboxyl group of D-Ala at position 4 and the amino group of the diamino acid at position 3, either directly or through a short peptide bridge (Vollmer et al., 2008a). Therefore, the chemical traits of peptidoglycan involve the presence of an unusual sugar (MurNAc), of γ-bonded D-Glu, of L–D (and even D–D) bonds and of nonprotein amino acids (e.g. A_2pm). These structural features are retrieved in all bacterial species known to date. However, a certain degree of variation exists either in the peptide stem, in the glycan strands or in the position or composition of the interpeptide bridge. Interspecies variation is the general case (Schleifer and Kandler, 1972). However, in the same species, there can be variations in the fine structure according to the growth conditions (growth phase, medium composition, intra/extracellular growth, presence of antibiotics) (Vollmer et al., 2008a).

3.1.2. The glycan strands in peptidoglycan

The glycan strands are formed by oligomerization of monomeric disaccharide peptide units (lipid II) by transglycosylation reactions. Secondary modifications in the glycan strands such as N-deacetylation, O-acetylation and N-glycolylation are frequently found (*N*-deacetylation is discussed below) (Vollmer et al., 2008a). In *S. aureus*, the glycan strands may contain either a MurNAc or a GlcNAc residue at the reducing end; the latter residue indicates that cleavage of the strand by an N-acetylglucosaminidase had occurred. In all Gram-negative and some Gram-positive species (e.g. *B. subtilis*), the glycan strands do not have a reducing (MurNAc or a GlcNAc) end but terminate with a 1, 6-anhydro MurNAc residue, which has an intramolecular ring from C1 to C6. It is not known whether the 1,6-anhydro MurNAc residues present in the sacculi have been formed during termination of glycan strand synthesis, or whether they are the result of degradation by lytic transglycosylases, or both (Vollmer, 2008). In species with high activity of glycan strand-cleaving enzymes (glucosaminidases and muramidases), the peptidoglycan may contain glycan strands with all possible combinations of GlcNAc and MurNAc residues at the ends (Schleifer and Kandler, 1972). There are only limited data on the average chain length and the chain length distribution of the glycan strands in different species. Remarkably, the average chain length of the glycan strands does not correlate with the thickness of the peptidoglycan layer, as there are Gram positive species with a thick cell wall with either short (*S. aureus*) or long (*B. subtilis*) glycan strands (Vollmer, 2008).

3.1.3. Variation in the peptide stem

The variations of the peptide stem can be divided into two categories: 1) those due to the specificity of the Mur ligases, the enzymes responsible for its biosynthesis, and 2) those occurring at a later step of the biosynthesis (Vollmer, 2008). The first amino acid of the peptide stem is added by the MurC ligase. In most bacterial species, this amino acid is L-Ala; in rare cases, Gly or L-Ser is added instead. The amino acid at the second position is added by the MurD ligase. In all species this enzyme adds D-Glu, the modifications encountered occurring at a later step (Vollmer, 2008). The greatest variation is found at position 3. The addition of the third amino acid is catalyzed by the MurE ligase. This amino acid is generally a diamino acid, either *meso*-A_2pm (Gram negative bacteria, Mycobacteria, Bacilli) or L-Lys (most Gram-positive bacteria). As for the second position, further modifications of the third amino acid occur posterior to MurE action (Vollmer, 2008). In most cases, the MurE enzyme is highly specific for the relevant amino acid; this has been demonstrated for the *meso*-A_2pm-adding and L-Lys-adding enzymes from *E. coli* and *S. aureus*, respectively. (Vollmer et al., 2008a). However, the MurE enzyme sometimes seems to be devoid of strict specificity, and this affects the final composition of peptidoglycan. In certain species of the genus *Bifidobacterium*, two diamino acids, L-Lys and L-Orn, are retrieved at the third position of the peptide stem (Schleifer and Kandler, 1972). Amino acids at positions 4 and 5 are added as a dipeptide, in most cases D-Ala-D-Ala. The synthesis of the dipeptide is carried out by the Ddl enzyme and its incorporation into the peptide stem by the MurF ligase. It has been established that the latter enzyme has a high degree of specificity for the C-terminal amino acid (Bugg et al., 1991; Duncan et al., 1990). This is complementary to the specificity of the former enzyme, which resides mainly on the N-terminal amino acid, and this constitutes a 'double-sieving' mechanism ensuring the synthesis of a pentapeptide stem ending mainly in D-Ala-D-Ala (Neuhaus and Struve, 1965). D-Ala is predominantly found at position 4 in all species. D-Lactate (D-Lac) or D-Ser is found at position 5 in strains endowed with natural or acquired resistance to vancomycin, the affinity of the D-Ala-D-Lac and D-Ala-D-Ser moieties for the antibiotic being much lower than that of the conventional D-Ala-D-Ala moiety (Healy et al., 2000). A certain proportion of Gly, presumably escaping from the double-sieving mechanism, is often found at position 4 or 5 in lieu of D-Ala (Vollmer et al., 2008b).

3.1.4. Other variations in peptidoglycan composition

Amidation, hydroxylation, acetylation, attachment of amino acids or other groups and attachment of proteins occur after the action of the Mur ligases. These modifications concern essentially positions 2 and 3. Most enzymes responsible for these modifications are still unknown (Vollmer, 2008; Vollmer et al., 2008b). Amidation of the α-carboxyl of glutamic acid (yielding D-isoglutamine) and the ε-carboxyl group of *meso*-A_2pm is very frequent. It has been shown in *Mycobacterium smegmatis* that lipid II is the substrate for amidation reactions; in fact, lipid II in this species appears as a mixture of non-, mono- and di-amidated molecules (Mahapatra et al., 2005). The hydrocarbon chain of D-Glu, *meso*-A_2pm or L-Lys is hydroxylated in some species. In the case of D-Glu, it was demonstrated that hydroxylation occurs after the cytoplasmic steps (Vollmer, 2008). In *Corynebacterium insidiosum*, where unsubstituted L-2,4-diaminobutyric acid is the substrate for MurE, the γ-amino group of the diamino acid is acetylated in the UDP-MurNAc-pentapeptide precursor and in peptidoglycan (Vollmer, 2008). In certain organisms, an amino acid or another amine-containing moiety, such as glycine (*Micrococcus luteus*, *Arthrobacter tumescens*), glycine amide (*Arthrobacter athrocyaneus*), D-alanine amide (*Arthrobacter* sp. NCIB9423), cadaverine (*Selenomonas ruminantium*) or *N*-acetylputrescine (*Cyanophora paradoxa*), is added to the α-carboxyl group at position 2 (Schleifer and Kandler, 1972; Vollmer, 2008). The peptide stem constitutes the point of covalent anchoring of some cell envelope proteins to peptidoglycan (Dramsi et al., 2008)

3.1.5. Variation in cross-linking

Most variations of the peptide moiety of peptidoglycan occur in its mode of cross-linkage and in the composition of the interpeptide bridge (Schleifer and Kandler, 1972; Vollmer, 2008). There are two main groups of cross-linkage. In the first group (3–4 cross-linkage), the cross-linkage extends from the amino group of the side-chain of the residue at position 3 of one peptide subunit (acyl acceptor) to the carboxyl group of D-Ala at position 4 of another (acyl donor). As mentioned above, this is the most common kind of cross-linkage. It can be either direct (most Gram-negative bacteria) or through an interpeptide bridge (most Gram-positive bacteria) (Vollmer, 2008; Vollmer et al., 2008b). The cross-linking reactions are catalyzed by the transpeptidase domain of penicillin-binding proteins, enzymes that have been studied extensively, in particular in human pathogenic bacteria (Sauvage et al., 2008). In the second

group (2–4 cross-linkage), found only among coryneform bacteria, the cross-linkage extends between the α-carboxyl group of D-Glu at position 2 of one peptide subunit (acyl acceptor) and the carboxyl group of D-Ala at position 4 of another (acyl donor). In this case, for the first subunit to be an acceptor, an interpeptide bridge containing a diamino acid must be present.

The size of the interpeptide bridge ranges from one to seven amino acid residues. Various amino acids are encountered: Gly, L-Ala, L- or D-Ser, D-Asx, L- or D-Glu, or others. The interpeptide bridges of 2–4 cross-links contain necessarily (but not exclusively) a diamino acid (L- or D-Lys, D-Orn, D-2, 4-diaminobutyrate). For many years, the important diversity of composition of the interpeptide bridges has contrasted with the poor knowledge of the branching enzymes responsible for their biosynthesis. In fact, it is only recently that some branching enzymes have been purified and characterized (Vollmer, 2008; Vollmer et al., 2008b). They can be divided into two groups according to the nature of the amino acid incorporated: 1) Glycine and L-amino acids are activated as aminoacyl-tRNAs and transferred to the precursors by a family of nonribosomal peptide bond-forming enzymes called Fem transferases (Mainardi et al., 2008). 2) D-Amino acids are activated as acyl phosphates by proteins belonging to the ATP-grasp family, which is composed of highly diverse enzymes that catalyze the ATP-dependent ligation of a carboxyl group to an amino or imino nitrogen, a hydroxyl oxygen or a thiol sulfur (Galperin and Koonin, 1997). The precursor substrate of the branching enzymes varies among species: lipid II for *S. aureus* enzymes, UDP-MurNAc-pentapeptide for *Weissella viridescens* FemX or both for *Enterococcus faecalis* enzymes (Vollmer, 2008). As for the main peptide chain, the interpeptide bridge can be further modified after its assembly. In *Enterococcus faecium*, where the Asl_{fm} enzyme ligates the β-carboxyl group of D-Asp to the UDP-Mur*N*Ac-pentapeptide, part of the α-carboxyl groups are subsequently amidated, thereby leading to a peptidoglycan composed of β-D-aspartyl and D-isoasparaginyl residues in similar amounts (Bellais et al., 2006).

Besides the diversity in the nature of cross-linkage, there is a considerable variation in the degree of cross-linkage, which varies from 20% in *E. coli* to over 93% in *S. aureus*. Translated in terms of muropeptide content, these figures mean that in *E. coli*, most peptidoglycan units appear as monomers (50%) or dimers (40%), higher oligomers being a minority (Glauner et al., 1988); conversely, in *S. aureus*, the percentage of monomers is low (<10%), most peptidoglycan units being present as oligomers (Snowden and Perkins, 1990).

3.1.6. Peptidoglycan N-deacetylation.

The muramidase lysozyme hydrolyzes the glycan strands of peptidoglycan between C1 of MurNAc and C4 of GlcNAc. In 1971, the presence of a high proportion of nonacetylated glucosamine (GlcN) residues in the peptidoglycan of *Bacillus cereus* was identified (Araki et al., 1971). A later study also identified nonacetylated muramic acid (MurN) residues in the peptidoglycan of *Bacillus anthracis* (Zipperle et al., 1984). The presence of these nonacetylated amino sugars accounted for the observed lysozyme resistance, as isolated cell walls could not be digested by lysozyme unless they were chemically N-acetylated by acetic anhydride. Other *Bacillus* species, including *B. anthracis*, *Bacillus thuringensis* and *B. subtilis* strain 168, contain deacetylated GlcN and MurN residues in their peptidoglycan (Vollmer, 2008). Nonacetylated GlcN was detected in the peptidoglycan of several other bacteria, such as *S. pneumoniae* (Ohno et al., 1982; Vollmer and Tomasz, 2000), *L. monocytogenes* (Boneca et al., 2007) and *L. lactis* (Meyrand et al., 2007) whereas nonacetylated MurN was detected in small quantities in *S. pneumoniae* (Vollmer and Tomasz, 2000) and *Micrococcus lysodeiktikus* (Hoshino et al., 1972). Deacetylated amino sugars appear to be widespread in peptidoglycan.

Deacetylated amino sugars are formed from the MurNAc and GlcNAc residues by peptidoglycan deacetylases. The supernatant of *B. cereus* cells contains an enzymatic activity capable of deacetylating GlcNAc residues in peptidoglycan. It was noted that the peptidoglycan deacetylase present in the crude fraction has a different biochemical property to that of the known GlcNAc-6-phosphate deacetylase (Araki et al., 1971). The deacetylation reaction is most likely to occur on polymerized peptidoglycan because deacetylated precursors have not been detected in species with deacetylated peptidoglycan, and known deacetylases have a predicted extracytoplasmic localization (Vollmer and Tomasz, 2000). Furthermore, inactivation of deacetylase genes is tolerated in different species with none or at most minor effects on cell growth (Boneca et al., 2007; Meyrand et al., 2007; Vollmer and Tomasz, 2000). Therefore, the absence of deacetylated amino sugars in the peptidoglycan does not appear to affect the peptidoglycan biosynthetic enzymes, the transglycosylases and transpeptidases in these mutants (Vollmer, 2008; Vollmer et al., 2008a).

The first gene encoding a peptidoglycan GlcNAc deacetylase, *pgdA*, was identified in *S. pneumoniae* (Vollmer and Tomasz, 2000). A *pgdA* mutant strain lacked deacetylated amino

sugars in its peptidoglycan. The amino acid sequences of the pneumococcal peptidoglycan deacetylase PgdA, rhizobial NodB chitooligosaccharide (nodulation factor) deacetylases and fungal chitin deacetylases are similar, which is not surprising because their substrates share a common structural feature. All three substrates (peptidoglycan, nodulation factor and chitin) have a backbone of β-1, 4-linked GlcNAc residues (In fact, MurNAc in peptidoglycan is the 3-lactyl ether of GlcNAc). These enzymes belong to carbohydrate esterase family 4 (CE4, PFAM01522), which includes peptidoglycan GlcNAc deacetylases (EC 3.1.1.-), rhizobial NodB chitooligosaccharide deacetylases (EC 3.5.1.-), chitin deacetylases (EC 3.5.1.41), acetyl xylan esterases (EC 3.1.1.72), and xylanases A, C, D and E (EC 3.2.1.8) (Vollmer, 2008). Members of this family hydrolyze either N-linked acetyl groups from GlcNAc residues (peptidoglycan deacetylases, rhizobial NodB chitooligosaccharide deacetylases, chitin deacetylases) or O-linked acetyl groups from O-acetylxylose residues (acetyl xylan esterases and xylanases A, C, D and E) of their substrates.

The carbohydrate esterase family CE4 contains over 800 members (Bornscheuer, 2002), many of which encode hypothetical polysaccharide deacetylases in bacterial genomes and have been reported to be metal ion-dependent. The amino acid sequence alone of CE4 family enzymes is not sufficient to determine the substrate (peptidoglycan, chitin, chitooligosaccharide or xylan) of a hypothetical deacetylase.. Therefore, both structural analysis of the peptidoglycan of a mutant strain lacking a hypothetical gene as well as biochemical characterization of the gene product are required to establish its functional activity. PgdA from *S. pneumoniae* is about twice the size of typical CE4 family proteins. PgdA has a predicted N-terminal membrane anchor followed by a large region of unknown function, and a C-terminal deacetylase domain (Vollmer and Tomasz, 2000). The crystal structure of a soluble form of PgdA was solved, showing a three-domain architecture, with the N-terminal domain from amino acids 46 to 160, a smaller domain from amino acids 161 to 268 and the deacetylase domain from amino acids 269 to 463 (Blair et al., 2005). Although there are significant topological differences between PgdA and PdaA from *B. subtilis*, the overall fold of the deacetylase domain and the catalytic core are similar in both structures. Structural analysis did not reveal possible *in vivo* functions for the N-terminal and middle domains of PgdA, both of which are absent in PdaA. PgdA contained a Zn^{2+} ion, complexed with two histidines and an aspartic acid, and this metal binding triad is conserved in members of the CE4 family. As expected from the crystal structure, the activity of PgdA is metal-

dependent and the addition of EDTA inactivates the enzyme (Blair et al., 2005). The enzyme activity was highest in the presence of Co^{2+}, but PgdA was also active in the presence of Zn^{2+}, Ni^{2+} or Fe^{2+}, whereas it showed little or no activity with Cu^{2+}, Cd^{2+}, Mg^{2+} or Ca^{2+}. PgdA deacetylated the middle GlcNAc residue of an artificial $GlcNAc_3$ substrate (Blair et al., 2005). Two other peptidoglycan GlcNAc deacetylases have been characterized biochemically, BC1960 and BC3618 from *B. cereus* (Psylinakis et al., 2005). Both were inactive against GlcNAc but were active against the oligosaccharides $GlcNAc_{2-8}$ and showed highest activity against $GlcNAc_4$. In these substrates, all GlcNAc residues were deacetylated except the one at the reducing terminus. Both enzymes deacetylated GlcNAc residues in a peptidoglycan fragment (GlcNAc NeuNAc -L-Ala-D-Glu) and in peptidoglycan from *Helicobacter pylori* and *B. cereus* (Psylinakis et al., 2005).

3.2. Cell-wall glycopolymers (CWG)

Within the fabric of the cell wall, Gram-positive bacteria contain additional CWGs. CWG structures are highly diverse and their functions are only partially understood (Weidenmaier and Peschel, 2008). CWGs differ with regards to their sugar building blocks and non-glycosyl residues. According to net charge, CWGs can be classified into zwitterionic, anionic and uncharged polymers. Polyanionic CWGs include, for example, pyruvylated polysaccharides from S-layer-protein-displaying bacilli and their relatives, teichuronic acids and succinylated lipoglycans. Uncharged, often branched CWGs are found, for example, in the cell walls of *Mycobacterium tuberculosis* and other actinobacteria (Weidenmaier and Peschel, 2008). Zwitterionic CWGs comprise WTA and lipoteichoic acids (LTA). For the needs of this thesis, certain characteristics of WTA and LTA will be reviewed. Since the current knowledge on *S. suis* WTA and LTA is insignificant, the following subsections will comprise, for the most part, data and conclusions from other organisms.

3.2.1. Wall Teichoic and Lipoteichoic Acids (WTA and LTA)

The presence of phosphate groups in the repeating units is the hallmark of teichoic acids (Figure 10). Due to the presence of these negatively charged phosphate groups as well modifications with free amino groups that are contained in residues such as D-alanine, teichoic acids have zwitterionic properties. WTA are most often formed by glycerol or ribitol groups that

are connected by phosphodiester bonds (for example, in *S. aureus*), although tetroses, hexoses or complex sugar combinations have also been described (for example, in *S. pneumoniae*). The polymers are peptidoglycan-attached via the linkage unit (Gro-P) $_{2 \text{ or } 3}$ManNAc-(β1-4)-GlcNAc-P (Gro-P, glycerol phosphate) to C6 of the MurNAc residues (Araki and Ito, 1989; Coley et al., 1978). To the best of our knowledge, WTA from *S. suis* have not been studied, perhaps because of technical difficulties. Indeed, attempts to obtain WTA from *S. suis* using common phenol extraction have failed (Elliott et al., 1977).

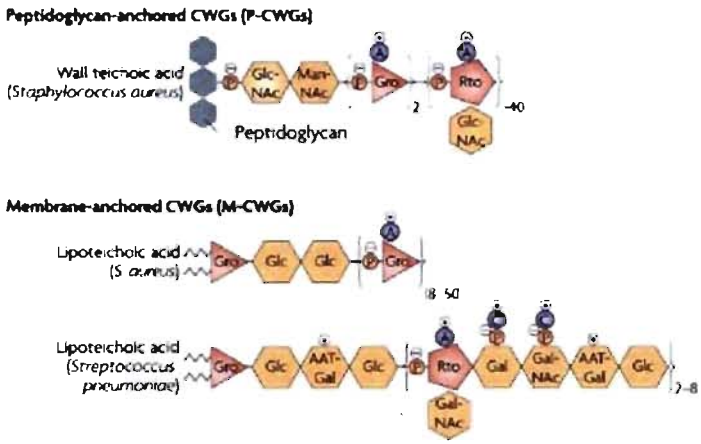

Figure 10. Structures of wall teichoic and lipoteichoic acids. Trioses, peptoses and hexoses are shown as triangles, pentagons and hexagons, respectively. Sugars, sugar-derived alcohols and sugar-derived acids are shown in yellow, orange and purple, respectively. Fatty acids are shown as zigzag lines. Non-glycosyl residues: A, D-alanine; C, choline; P, phosphate; Glycosyl residues: AAT-Gal, 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose; Ara, arabinose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; Gro, glycerol; Ins, inositol; Man, mannose; ManNAc, *N*-acetylmannosamine; Rha, rhamnose; Rto, ribitol. Adapted from Weidenmaier and Peschel, 2008.

The membrane-anchored LTA are less diverse than WTA. LTA polymers are usually formed by Gro-P or ribitol phosphate repeating units and are connected to glycolipids. However, more complicated structures have been described; for example, in pneumococci, the LTA repeating units of which are identical to pneumococcal WTA (Neuhaus and Baddiley, 2003). The 1,3-glycerol-phosphate (-Gro-P-) and 1,5 D-ribitol-phosphate monomers are joined via anionic phosphodiester linkages to form linear chains (Baddiley, 1972). In many LTAs composed of poly (Gro-P) the polymer (which can vary from 5 to 50 units, depending on the species and/or growth conditions) is attached to the C6 of the nonreducing glucosyl of the glycolipid anchor (Neuhaus and Baddiley, 2003). In staphylococci, *Bacillus* sp, and most streptococci (one important

exception is *S. pneumoniae*) the glycolipid is Glc(β1-6)Glc(β1-3)(gentiobiosyl)diacyl-Gro (Neuhaus and Baddiley, 2003). It has been reported that the *S. suis* LTA has a structure differing from that described above, possibly in the attachment of its glucosyl substituents (Elliott et al., 1977). Precipitation reactions between *S. suis* LTA and Group D antisera were specifically inhibited by glucose, but extracts from *S. faecalis* and *S. faecium* were not. This fact may indicate a monosaccharide glucosyl substituent in teichoic acid from *S. suis* instead of the disaccharide previously postulated as the glucosyl substituent in the teichoic acid of *S. faecalis.* With the exception of these findings, very little is known regarding LTA from *S. suis*.

Most teichoic acids are decorated with additional sugars and amino acids. WTA and LTA from low G+C Gram positive bacteria are usually modified with D-alanine (Neuhaus and Baddiley, 2003), whereas acetyl, glutamyl and lysyl modifications have been described in WTA from high G+C Gram positive bacteria (Neuhaus and Baddiley, 2003; Weidenmaier and Peschel, 2008). WTAs and LTAs make up a polyanionic network or matrix that provides functions relating to the elasticity, porosity, tensile strength, and electrostatic steering of the envelope. The D-alanyl esters of these polymers, resulting from a single D-alanine incorporation system encoded by the *dlt* (for "D-alanyl-LTA") operon (see below) (Neuhaus et al., 1996), constitute important substituents for modulating the properties of the envelope in many species. For this reason, knowledge of these ester residues is essential for understanding the functions of teichoic acids in bacterial physiology as well as in host-mediated responses. A wide structural diversity of WTAs exists among Gram positive bacteria. Some of this diversity is confined to the presence and nature of the glycosyl substituents, D-alanyl esters, and repeating units (monomers) (Baddiley, 1972; Neuhaus and Baddiley, 2003).

3.2.2. D-Alanylation of LTA

Without exception, the alanyl esters of teichoic acids are of the D-configuration (Armstrong et al., 1959). The D-alanyl ester content in WTAs and LTAs is highly variable. The molar ratios of D-alanine to P (degree of D-alanylation) in LTA from a variety of species vary from not detectable to 0.88. WTAs generally have a lower ratio of D-alanine to P than do LTAs. For example, the ratio of D-alanylation (WTA/LTA) in *S. aureus* is 0.75 (Neuhaus and Baddiley, 2003). The D-alanyl ester contents of LTAs and WTAs of *S. aureus* and *B. subtilis* are a function of the pH of the growth medium (MacArthur and Archibald, 1984). Interestingly, *S. mutans* deficient in D-

alanine esters loses its acid tolerance response (Boyd et al., 2000). In addition to medium pH, the degree of D-alanylation is a function of the temperature as sublethal heating of *S. aureus* resulted in a loss of 65% of the D-alanyl ester content of teichoic acids (Neuhaus and Baddiley, 2003). The degree of D-alanylation is also affected by growth on media containing increasing concentrations of NaCl (Neuhaus and Baddiley, 2003).

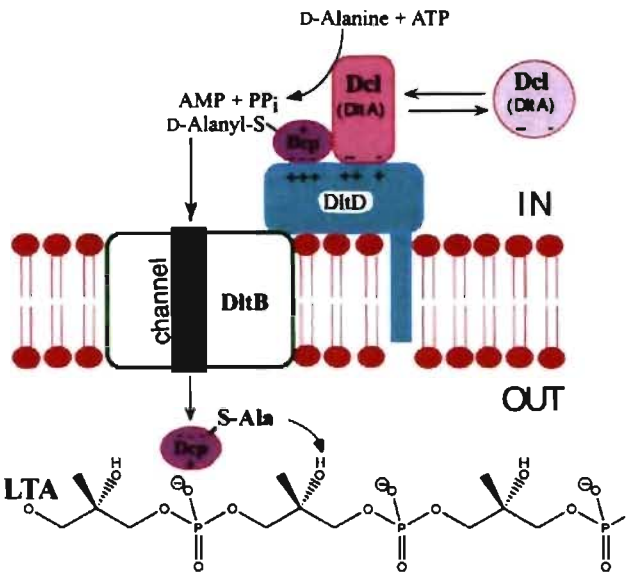

Figure 11. Model for the incorporation of D-alanyl ester residues into LTAs.

DltD provides binding sites for DltC (Dcp) and DltA (Dcl) on the cytoplasmic leaflet. DltB provides a putative channel for the secretion of D-alanyl-Dcp to the periplasm where D-alanylation occurs. Adapted from Neuhaus and Baddiley, 2003.

Although biosynthetic pathways for WTA and LTA differ, the D-alanyl-ester substituents of WTA are derived from those of D-alanyl-LTA (Neuhaus and Baddiley, 2003), consistently with a single *dlt* operon encoding the machinery for D-alanine incorporation(Perego et al., 1995). One of the remarkable features of the D-alanyl esters of teichoic acids is their dynamic turnover. For example, a $t_{1/2}$ of 37 min was observed for D-alanyl-LTAs in *S. aureus* growing at pH 7. It was postulated that the turnover is an enzyme-catalyzed process. In toluene-treated cells of this organism, the D-alanyl esters are lost from LTA and replaced by the ATP-dependent incorporation of new ones. "Reesterification" of vacant sites in LTA and WTA maintains the D-alanyl ester content of both teichoic acids. The rate of D-alanyl LTA synthesis is correlated with the rates of ester loss that occurs through transfer to WTA and through "base-catalyzed"

hydrolysis. Thus, from both *in vitro* and *in vivo* pulse-chase experiments, it was concluded that the D-alanyl esters of LTA are the precursors of those in WTA (Neuhaus and Baddiley, 2003).

Isolation of the gene encoding the activating enzyme (*dltA*) from *L. rhamnosus* ATCC 7469 provided the key for identifying the role of this enzyme in D-alanine incorporation (Heaton and Neuhaus, 1992). The enzyme (DltA, Dcl) is a member of a large protein family that both activates and transfers amino or fatty acids via a 4'-phosphopantetheine prosthetic group of a carrier protein or coenzyme A (Neuhaus and Baddiley, 2003). A heat-stable protein encoded by *dltC* contains this prosthetic group and functions as the D-alanyl carrier protein (DltC, Dcp) (Heaton and Neuhaus, 1994). Thus, DltA not only activates D-alanine but also ligates the activated ester to the 4'-phosphopantetheine prosthetic group of the carrier protein. Therefore, the activating enzyme is now designated D-alanine:DltC ligase (AMP forming) (Neuhaus and Baddiley, 2003). In addition to *dltA* and *dltC*, the operon contains two additional genes, *dltB* and *dltD*. The hydropathy profile of DltB shows a pattern of 12 putative membrane-spanning domains (Neuhaus et al., 1996). While it is not established, it has been suggested that one of the functions of DltB is the secretion of unfolded D-alanyl-DltC (Neuhaus and Baddiley, 2003). The reversibility of the thermal denaturation of DltC is consistent with this suggestion (Volkman et al., 2001). Comparison of DltB with a variety of O-acyltransferases identified two conserved motifs that may also link this transport protein to a superfamily of membrane-bound *O*-acyltransferases (Hofmann, 2000). Whether DltB functions in the actual secretion of D-alanyl-DltC, whether it functions as an acyltransferase, or whether it is bifunctional is not known. The membrane protein, DltD, functions in the selection of the correct carrier protein, DltC, for ligation with D-alanine and in the hydrolysis of mischarged D-alanyl-acyl carrier proteins (Debabov et al., 2000). It has been proposed that DltD facilitates the binding of DltC and DltA for ligation of DltC with D-alanine and that DltD functions in the final esterification step (Neuhaus and Baddiley, 2003). The transfer of the D-alanyl residue from D-alanyl-Dcp to LTA requires only that the acceptor LTA be membrane associated (Heaton and Neuhaus, 1994). None of the acyl carrier proteins involved in fatty acid metabolism replace the requirement for Dcp, even though Dcl ligates D-alanine to acyl carrier proteins in the absence of DltD (Heaton and Neuhaus, 1994). Figure 12 summarizes the current proposed mechanism of D-alanyl ester incorporation to LTA.

3.2.3. Roles of WTA and LTA in attachment, colonization and virulence

S. aureus mutants that lack WTA are profoundly inhibited in their ability to bind to nasal epithelial cells, and were unable to colonize the nares of cotton rats in an experimental nasal colonization model (Weidenmaier, 2004, 2008). WTA seems to play a similar role in *S. aureus* adhesion to endothelial cells (Weidenmaier, 2005b). The impact of WTA deficiency is markedly increased under flow conditions in which the bacterial cells move over the host cell surface with sufficient velocity. *In vitro* and *in vivo* evidence indicate that WTA contributes to a similar extent to *S. aureus* adhesion to epithelial and endothelial cells as staphylococcal proteinaceous adhesins that bind, for example, to fibronectin or fibrinogen, and a lack of WTA does not seem to influence adhesin activities (Weidenmaier, 2005b).

LTA has also been implicated in adhesion to host cells. It has been shown that *S. suis* LTAs are important for adhesion of this bacterium to porcine BMEC. Indeed, inhibition of the adhesion of *S. suis* to this cellular type can be obtained by pre-incubation of PBMEC with purified LTA (Vanier et al., 2007). In other species, inactivation of the *dlt* operon resulted in a significantly reduced ability of *S. aureus* to bind to epithelial and endothelial cells and cause infections (Weidenmaier, 2005a). In addition, formation of D-alanyl-LTAs has been shown to be required to resist the action of antimicrobial peptides in *L. monocytogenes*, *S. aureus*, *S. pneumoniae*, GAS and GBS (Abachin et al., 2002; Kovacs et al., 2006; Kristian et al., 2005; Poyart et al., 2003; Weidenmaier et al., 2005). In addition, virulence of *S. aureus* mutants deficient in LTA D-alanylation was severely impaired in rabbit models of infection (Weidenmaier et al., 2005). Similar observations have been made upon deletion of the *dlt* operon in *L. monocytogenes* (Abachin et al., 2002) and GAS (Kristian et al., 2005).

III. MATERIALS, METHODS AND RESULTS

ARTICLE I

"Use of Selective Capture of Transcribed Sequences to Identify Genes Preferentially Expressed by *Streptococcus suis* upon Interaction with Porcine Brain Microvascular Endothelial Cells."

Nahuel Fittipaldi, Marcelo Gottschalk, Ghyslaine Vanier, France Daigle and Josée Harel.

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Details on the role of the candidate in the conception of the article: I substantially contributed to research design, performed research, analyzed data and wrote the paper.

Use of Selective Capture of Transcribed Sequences to Identify Genes Preferentially Expressed by *Streptococcus suis* upon Interaction with Porcine Brain Microvascular Endothelial Cells

Nahuel Fittipaldi [1, 2], Marcelo Gottschalk [1, 2], Ghyslaine Vanier [1,2], France Daigle [1,3] and Josée Harel [1,2]*.

Running title: Candidate *S. suis* virulence factors identified using SCOTS

Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine[1], Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Qc, J2S 7C6, Canada[2], Département de Microbiologie et Immunologie, Université de Montréal, Montreal, Qc, H3C 3J7, Canada[3].

* Corresponding author. Mailing address: GREMIP, Faculté de Médecine Vétérinaire, Université de Montréal, CP 5000, St-Hyacinthe, Qc, J2S 7C6, Canada. Phone: (450) 773-8521, ext. 1-8233. Fax: (450) 778-8108. E-mail: [redacted]

Abstract

Using the selective capture of transcribed sequences (SCOTS), we identified 28 genes preferentially expressed by the major swine pathogen and zoonotic agent *Streptococcus suis* upon interaction with porcine brain microvascular endothelial cells. Several of these genes may be considered new *S. suis* virulence factor candidates. Results from this study demonstrate the suitability of SCOTS for the elucidation of gene expression in streptococcal species and may contribute to a better understanding of the pathogenesis of *S. suis* infections.

Main Text

Streptococcus suis is a Gram-positive bacterium responsible for, among other diseases, meningitis and septicemia in swine (14). *S. suis* is also a zoonotic agent: Many cases of humans *S. suis* infections have been reported in different Asian and European countries as well as in New Zealand, Australia, Argentina and Canada (25). Very recently, the first case of human meningitis caused by *S. suis* was recorded in the United States (43). Indeed, *S. suis* is increasingly becoming a public health concern. For instance, during a recent outbreak in China more than 200 cases of human *S. suis* infection were reported, 39 of which resulted in death (33, 45). Despite increasing research in recent years, knowledge on the pathogenesis of *S. suis* infection remains limited (11, 14). Only the capsular polysaccharide (CPS) and a recently described serum opacity-like factor have been shown to play a critical role in the pathogenesis of the infection (3, 14). Proposed putative virulence factors such as the suilysin, the extracellular protein factor (EF) and the muramidase-released protein (MRP), although associated to virulence, have been found to be nonessential factors (6, 14). Other determinants, such as a fibronectin/fibrinogen-binding protein were found to be partially involved in virulence (6, 14) while the actual role of some other virulence candidates (e.g., the cell wall and several putative adhesins and proteases) in the pathogenesis of *S. suis* infection remains to be verified (11, 14).

S. suis needs to attain the central nervous system (CNS) in order to cause meningitis in swine. It has been suggested that this pathogen might reach the CNS by crossing the porcine blood brain barrier (BBB) by transcytosis through porcine brain microvascular endothelial cells (PBMEC) and/or porcine choroid plexus epithelial cells (PCPEC), as well as by disruption of the barrier caused by toxic effects on BBB forming cells (11, 36). Support for these mechanisms has been provided by recent studies showing that *S. suis* is able to affect the viability of PCPEC through necrotic and apoptotic mechanisms (37) and to adhere to and to invade *in vitro*-cultured PBMEC (38). However, little is known on the molecular means by which *S. suis* accomplish these processes.

The selective capture of transcribed sequences (SCOTS) is a PCR-based RNA analysis method that offers several advantages in comparison to other genomic approaches such as the *in vivo* expression technology (IVET) or the signature tagged mutagenesis (STM) (29). In fact, SCOTS directly identifies bacterial genes rather than promoter regions and is not confounded by

polar effects when genes are arranged in polycistronic operons (29). The SCOTS approach has been used with success in many Gram-negative bacteria as well as in *Mycobacterium tuberculosis* and *Listeria monocytogenes* (5, 10, 13, 22). In this work, we used the SCOTS approach in order to identify genes preferentially expressed by *S. suis* during its interactions with cells of the BBB, a process that might be essential for the pathogenesis of the meningitis caused by this pathogen.

Experimental model and bacterial transcriptome recovery.

S. suis serotype 2 highly virulent strain 31533 (38) and the PBMEC line PBMEC/C1-2 (34) were used in this study. PBMEC were grown in Primaria 6-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) using IF culture medium (a mixture of 1:1 Iscove's modified Dulbecco's and Ham's F-12 media, Invitrogen, Burlington, ON, Canada) as previously described (38). *S. suis* was grown in Todd-Hewitt broth (Becton Dickinson, Sparks, MD) for 16 h at 37°C, harvested by centrifugation, washed twice in phosphate-buffered saline, pH 7.3 and resuspended in fresh IF culture medium at 10^6 CFU/ml. Confluent monolayers of PBMEC (at 3 x 10^6 cells per well) were inoculated with 3 ml of this bacterial suspension (MOI=1). Plates were centrifuged at 800 x *g* for 10 min and incubated for 4 h at 37°C with 5% CO_2. After incubation, actual *S. suis* adhesion to and invasion of PBMEC were verified in selected wells and found to be in agreement with reported values (38) (data not shown). For identification of genes transcribed during interaction, total RNA from *S. suis* infected PBMEC cells was prepared from 24 independent P6-wells using RNAwiz (Ambion, Austin, TX) according to the manufacturer's instructions. Total RNA from *S. suis* grown under the same conditions but without cells (mock-infection) was prepared from 5 P6-wells. Samples were treated with TurboDNAse (Ambion), and absence of contaminating DNA was verified by PCR using primers AROA-F and BA9 (Table 1), which target the *aroA* gene. RNA was quantified by measurement of absorbance at 260 nm and its integrity was verified by visualization on 1% denaturing agarose gels.

Selective capture of transcribed sequences

Five µg of total RNA prepared from both infection and mock-infection samples were reverse transcribed by random priming using SuperScript II (Invitrogen). Primers CELL-RNA or MOCK-RNA, with a defined terminal sequence at the 5'-end and a random nanomer at the 3'-

end were used (Table 1). Thereafter, cDNA sequences corresponding to bacterial mRNAs were selectively captured from the mixture of total PBMEC-*S. suis* RNAs or total *S. suis* RNAs by performing 3 rounds of SCOTS as previously described (5). Briefly, samples were normalized by self hybridization, then hybridized overnight at 68°C to biotinylated genomic *S. suis* 31533 DNA that had previously been blocked using PCR-generated DNA representing 16S and 23S *S. suis* rRNA sequences (primers RDNA-F and RDNA-R, Table 1). Bacterial cDNAs were then separated using streptavidin-coupled magnetic beads. After elution, cDNAs were PCR re-amplified using primers CELL-PCR or MOCK PCR (Table1), that correspond to the defined sequences added during reverse transcription and specific to each condition. Sequences preferentially transcribed by *S. suis* upon interaction with PBMEC were obtained after three rounds of enrichment carried out as previously described (5). Briefly, cDNAs obtained during PBMEC interaction were subjected to the procedure outlined above, but this time the biotinylated genomic *S. suis* DNA used for capture had previously been pre-hybridized with DNA sequences corresponding to 16S and 23S *S. suis* rRNA and cDNAs from the mock infection. Resulting interaction-specific cDNAs were cloned into vector pCR4 (TOPO TA Cloning kit, Invitrogen) and sequenced. DNA sequences were determined at the DNA Sequencing Facility of the University of Maine (Orono, ME), on a 373A DNA Sequencing System (Applied Biosystems, Foster City, CA).

Identification of preferentially expressed genes

The BLAST software package was used to determine sequence homologies in the GenBank databases (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence comparison was also performed against sequence data produced by the *S. suis* Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_suis) for European strain P 1/7 and at the Joint Genome Institute Microbial Genomics (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=strsu&advanced=1) for North American strain 89-1591. We report in this study the identification, using SCOTS, of 28 genes as being preferentially expressed by *S. suis* upon interaction with PBMEC. These genes can be divided into 8 functional groups: metabolism/housekeeping, cell envelope, secreted proteases, cell division/replication, regulatory, protein sorting and transport/binding related genes, as well as genes with unknown function. To the best of our knowledge, none of the identified genes has ever before been associated to the *S. suis* pathogenesis of infection. Similar to other studies on host-pathogen interaction (2, 5, 31), most of the genes identified by SCOTS in this study are putatively involved

in metabolic/housekeeping functions and do not encode "genuine" virulence factors. However, identification of these genes may be of importance, since new information about the metabolism of *S. suis* is rendered that may eventually prove useful for vaccine development. On the other hand, some genes identified using SCOTS in this study are known to be important for the virulence of other Gram-positive bacteria (including at least three different streptococcal species). The relevance of these genes will be discussed below. For all other genes identified in this study, putative functions as well as references to publications describing *in vivo* expression and/or involvement in virulence in other organisms are listed in Table 2.

Validation of SCOTS results by quantitative PCR

The SCOTS approach, as used in this study, should result in the identification of genes that are upregulated by *S. suis* upon interaction with PBMEC (5). Therefore, to validate our SCOTS results, we measured by quantitative PCR (q-PCR) the level of expression of random selected genes on a new series of infection replicates. Infection of PBMEC, mock-infections and RNA extraction from both samples was performed as described above. cDNAs were synthesized in triplicate by using SuperScript II with random hexamers (Roche, Laval, Qc, Canada). Q-PCR was performed by using the QuantiTect SybrGreen PCR Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. For each sample, a no-reverse transcription reaction was run as a control. Primers are described in Table 1. For each q-PCR run, the calculated threshold cycle (C_t) was normalized to the C_t of the internal control *rpoD* gene amplified from the corresponding sample, and the fold change was calculated using the 2-[Delta][Delta] C_t method, as described (23). Results of q-PCR analysis for these selected genes showed that they were indeed upregulated by *S. suis* upon interaction with PBMEC (Figure 1), with fold changes ranging from 2.18 to 10. The gene *aroA*, known to be expressed in equal amounts in both conditions (our unpublished results), was also used.

Genes involved in cell envelope modification

As stated above, some genes identified by SCOTS might be considered, based on their functions in other organisms, as potential virulence factor candidates for *S. suis*. For instance, the gene *ssu0597* (*dltB*) belongs to an operon comprising four genes, *dltA, dltB, dltC*, and *dltD*, which is present in all genomes of low-G+C bacteria determined so far (28). In all species where

this operon has been studied, all four genes are required to catalyze the incorporation of D-alanine residues into the lipoteichoic acids (LTAs). D-alanylation of LTAs allows Gram-positive bacteria to modulate their surface charge, to regulate ligand binding and to control the electromechanical properties of the cell wall (28). In addition, formation of D-alanyl-LTAs is required to resist the action of antimicrobial peptides in *L. monocytogenes, Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes* (GAS) and *Streptococcus agalactiae* (GBS) (1, 20, 21, 30, 42). Besides, virulence of mutants deficient in D-alanylation of LTAs of GBS, *L. monocytogenes* or *S. aureus* was severely impaired in the murine or rabbit models of infection (1, 30, 42). The D-alanylation of *S. suis* LTAs has not yet been documented. However, it is known that wild-type *S. suis* LTAs are important for adhesion of this bacterium to PBMEC. Indeed, inhibition of the adhesion of *S. suis* to this cellular type can be obtained by pre-incubation of PBMEC with purified LTA (39). From our SCOTS results, it might be hypothesized that *S. suis* might be able to modulate the degree of D-alanylation of its LTAs by upregulation of its *dlt* operon upon interaction with PBMEC. Further studies focusing on this operon of *S. suis* are underway to evaluate this hypothesis. However, it is interesting to note that in some Gram-positive pathogens it has been shown that D-alanyl-LTAs contribute to adhesion to and invasion of various cell lines and that these steps may depend on a high ratio of D-alanine to glycerol/ribitol phosphate in their LTAs (1, 21, 30, 42).

The main clinical feature of *S. suis* is meningitis, and this bacterium is often isolated from the CSF of animals or patients with meningitis (14). On the other hand, it has been reported that patients suffering from meningitis present increased titers of lysozyme in their CSF (19). As shown in this study, *S. suis* differentially expressed a gene (*ssu1448*) highly homologous to *S. pneumoniae pgdA*, which encodes a peptidoglycan N-acetylglucosamine deacetylase. Peptidoglycan is an essential component of the bacterial cell wall and an important target for the innate immune system. Peptidoglycan modification by deacetylation seems to be important for Gram-positive pathogens. Indeed, pneumococci in which *pgdA* was inactivated became hypersensitive to the action of lysozyme (41) and showed reduced virulence in a murine model of infection (40). In addition, it has very recently been reported that a *pgdA* mutant strain of *L. monocytogenes* was impaired in its ability to induce disease in the murine model of infection and that the *pgdA* gene was required by this species to resist the host innate immune response mediated by lysozyme (4). In this regard, it may be of interest to further evaluate the hypothesis

that *S. suis*, through the action of the *pgdA* gene product, has the ability to modify its peptidoglycan by deacetylation and, therefore, resist a host innate response mediated by this enzyme. On the other hand, it is intriguing that, in our *in vitro* model, where the immune response of the host would not be as relevant as in the *in vivo* situation, the *pgdA* gene was found to be highly upregulated. However, it has been proposed that, *in vivo*, *S. suis* might gain access to the CNS by transcytosis across PBMEC (38). It might therefore be plausible that during the interactions with PBMEC, in addition to genes required for adhesion/invasion of these cells, *S. suis* also upregulates genes required for the steps immediately following the BBB crossing. Further studies are required to evaluate this hypothesis.

Identification of a putative pilus island in *S. suis*

Pili in several Gram-positive bacteria have recently been described and it has been proposed that they may play an important role in virulence (35). For instance, in GBS, pili participate in adhesion to human epithelial cells (7) and their role in adhesion to extracellular matrix (ECM) proteins has been suggested (27). In this work we identified a gene (*ssu0424*) putatively encoding a signal peptidase homologous to the LepB-type signal peptidases of Gram-negative bacteria. An homologous LepB-type signal peptidase is the first gene in the GBS pilus island 2b (PI-2b), one of the 3 identified pili islands in this species (35). The GBS PI-2b contains 5 other downstream genes, encoding two LPXTG proteins (suggested to be an ancillary protein and the main pilus subunit), a class C sortase, a third LPXTG protein (ancillary protein) and a second class C sortase (35) (Figure 2). The presence of thin pilus-like structures on the surface of *S. suis* has been revealed by electron microscopy (15). Interestingly, analysis of data from the two *S. suis* sequencing projects strongly suggests that *S. suis* possesses a truncated version of this pilus island. As a matter of fact, in *S. suis* sequenced strains P 1/7 and 89-1591, two genes encoding LPXTG proteins (highly homologous to the ancillary and main pilus subunits of GBS, respectively) and a gene encoding an undescribed putative class C sortase-like protein are found downstream the LepB signal peptidase that was identified by SCOTS (Figure 2). Although the *S. suis* putative pilus island lacks the last 2 genes in comparison to that of GBS, the similarity of the genetic organization, the strong homology showed by the LPXTG proteins to the main and ancillary pilus proteins of this latter species and the current proposed mechanism for pili formation in Gram-positive bacteria (7, 27), suggest that a pilus might be formed by the gene products of this island. In addition, we speculate that this pilus might participate in *S. suis*

adhesion to or invasion of PBMEC. In fact, pili have been very recently shown to be important for GBS adhesion to and invasion of human BMEC (26). Although functional analysis of this *S. suis* putative pilus island is needed to fully evaluate this hypothesis, it is interesting to note that the LepB signal peptidase was found to be highly upregulated by q-PCR (4-fold change) upon interaction of *S. suis* with PBMEC (Figure 1). Additionally, we identified in this study the gene *ssu0453*, previously named *srtE* and encoding one of the 4 class C sortases already described in *S. suis* (8, 29). In GBS and GAS models of pili assembly, class C sortases have been proposed to catalyze the covalent polymerization of the pilin subunits encoded by genes within the pilus island bearing the class C sortases (7, 27). However, previous work on *S. suis* (29), as well as analysis of sequenced strains P 1/7 and 89-1591, indicate that the *S. suis srtE* gene is not flanked by genes encoding LPXTG proteins and, as such, it does not seem to be part of a pilus island. Therefore, a putative participation of *S. suis ssu0453/srtE* in pili formation following the proposed model is unlikely. However, it might be interesting to evaluate whether this sortase might be required for, or contribute to, the assembly of pilin subunits encoded by the island described in this work or by other, yet unidentified islands.

Interaction of *S. suis* with ECM proteins

It has been shown that *S. suis* is able to interact with ECM proteins (9). As well, *S. suis* has the ability to degrade ECM proteins through the upregulation of metalloproteinase 9 production by human macrophages (16), that may result in tissue destruction and BBB disruption. However, the direct ability to degrade ECM proteins has not yet been demonstrated for *S. suis*. Interestingly, one of the genes identified by SCOTS (*ssu0457*) is a putative collagenase, which, in sequenced strains P 1/7 and 89-1591, is located upstream of a gene putatively encoding a second collagenase, in an operon-like organization. It has been suggested that the impairment of BBB barrier function during infection with different *S. suis* strains may depend on proteases produced by this pathogen (17). It is thus tempting to speculate, even if we lack evidence regarding its exact function, that upregulating the expression of the collagenase identified in this work upon interaction with PMEC might, *in vivo*, be useful to increase the permeability of the BBB and therefore contribute to the migration of *S. suis* to the CNS.

Suitability of the SCOTS approach to elucidate gene expression in *S. suis*

The SCOTS approach has been used with success in several bacterial species (5, 10, 13, 22). To the best of our knowledge, this is the first report of its use in a streptococcal species. Results presented here clearly demonstrate that SCOTS is also suitable for the elucidation of gene expression in streptococci and particularly in organisms like *S. suis*, for which very few molecular tools exist. Indeed, with the exception of the present study, only one genomic approach has been used to study this pathogen (32). In that work, an adapted *in vivo* expression technology (IVET) approach identified several *S. suis* iron-induced and/or *in vivo* (porcine infection model)-expressed genes (32). However, all the genes identified in that study were also expressed *in vitro* under standard laboratory growth conditions. These results can be explained by the absence of promoter sequences exclusively expressed under the conditions tested (32). However, since a plasmid-based instead of an integrative promoter trap system was used, results might as well be explained by the inability of that system to detect *in vitro* silent genes due to gene dose effects (32). On the other hand, using SCOTS we clearly showed condition-specific differences in *S. suis* gene expression. In fact, currently, SCOTS may be considered the only approach available for the direct study of global differential gene expression in *S. suis*. Despite the fact that IVET and SCOTS have identified the same genes in some cases (31), there were no overlapping genes between the IVET and SCOTS *S. suis* studies. This was not surprising, however, since only a small number of genes were identified in either study, and, more importantly, the experimental conditions used were essentially different. Therefore, in this study, the use of the SCOTS approach resulted in original insights on the molecular mechanisms that this pathogen might use to cross the BBB. Indeed, the identification of the 28 genes preferentially expressed upon interaction of *S. suis* with PBMEC, several of which show a great potential as virulence factor candidates, may result in a better understanding of how this pathogen causes meningitis. In addition, extending the SCOTS analysis to identify transcriptional differences at different *in vivo* localizations (i.e., brain, heart, tonsils) as well as at different stages of infection may lead to the comprehension of the mechanisms of disease progression and provide clues to its prevention.

Addendum in proof

While this work was under revision an article was published (44), describing the use of the STM approach to study genes important for the virulence of *S. suis* in a pig model of infection. The gene *ssu0457* reported in the present article was also found in that study.

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Article I Tables

Table II. Article I, Table 1. Oligonucleotide primers used in this study.

Primer name	Sequence (5'-3')
AROA-F	AACGTGACCTACCTCCGTTG
AROA-R	CGGTCATCGTAGAATTCGAGT
CELL-RNA	GACACTCTCGAGACATCACCGGTACCNNNNNNNNN
MOCK-RNA	CTTAGCCACTACGTGCGGATCCAGACNNNNNNNNN
RDNA-F	GGCTCAGGACGAACGCTG
RDNA-R	GCTAAGCGACTACCGTATCT
MOCK-PCR	CTTAGCCACTACGTGCGGATCCAGAC
CELL-PCR	GACACTCTCGAGACATCACCGGTACC
RPOD-F	TCTTTCAAATACATGCGGACTG
RPOD-R	ATTCCATTTACGCTTGATGCTG
SSU0424-F	AATCAAAGATTGGACGAGCC
SSU0424-R	CAATCCATCCCAATTCAGACAG
SSU0870-F	GGTATCATGAATACGGACGAAG
SSU0870-R	GAATGGATGGGCAATGAGAG
SSU0067-F	ATCAATCATCAAGGGATGCG
SSU0067-R	GATAGCCACCTCTTTTTCCAC
SSU1448-FQ	TTCTCTCTGTACTTGCTCCC
SSU1448-RQ	GGTCGCTCTAACCTTTGATG
SSU0457-F	ACCCAGATAGCCACTATTCC
SSU0457-R	CTGATCATAAGTGAAGTCGCC
SSU0597-F	TGCGTCTGGTTAAGACTTTG
SSU0597-R	GTTCTTGCCCAGCTTTTTTTC

Table III. Article I, Table 2. Genes differentially expressed by *S. suis* upon interaction with porcine brain microvascular endothelial cells identified using selective capture of transcribed sequences.

Clone	Gene [1]	Putative function [Organism]	Genbank ID	Reference
Metabolism / Housekeeping				
D3C3	ssu0707	Putative exonuclease RexB [*S. suis* 89/1591].	ZP_00874950	
2H6	ssu0767	1-phosphofructokinase [*S. suis* 89/1591].	ZP_00875124	(24)
2G7	ssu1411	Aminotransferase, class I and II [*S. suis* 89/1591]	ZP_00875661	
D3G2	ssu1527	Aminodeoxychorismate lyase-like protein [*S. suis* 89/1591].	ZP_00876086	
1B7	ssu1445	Uridine kinase [*S. thermophilus* CNRZ1066].	AAV_62804	
D1B7	ssu0844	Haloacid dehalogenase-like hydrolase [*S. suis* 89/1591].	ZP_00874652	
1G10	ssu1044	Ribonucleoside-diphosphate reductase [*S. suis* 89/1591].	ZP_00875241	
D1H10	ssu1159	Ribosome recycling factor [*S. suis* 89/1591].	ZP_00875081	
1C7	ssu0870	Nucleotidyl transferase [*S. suis* 89/1591]	ZP_00874394	(12)
2F10	ssu0871	Glucose-1-phosphate adenylyltransferase [*S. suis* 89/1591].	ZP_00874395	
1A11	ssu0764	tRNA (guanine-N1-)-methyltransferase [*S. suis* 89/1591].	ZP_00875121	
Cell envelope				
2A11	ssu0597	Membrane bound O-acyl transferase, DltB [. *suis* 89/1591].	ZP_00875261	(12)
2A8	ssu1184	D-alanine-D-alanine ligase [*S. suis* 89/1591].	ZP_00875052	(18)
1H9	ssu1448	Peptidoglycan polysaccharide deacetylase PgdA [*S. suis* 89/1591]	ZP_00876135	(40, 41)
1E9	ssu1487	VanZ like protein [*S. suis* 89/1591].	ZP_00875572	(12)
D1G11	ssu1114	Glycosyl transferase, group 1 [*S. suis* 89/1591].	ZP_00875224	
Regulatory				
D2D4	ssu0869	Putative transcriptional regulator, LysR family [*S. pneumoniae* TIGR4].	AAK74821	
Protein sorting				
1C11	ssu0424	Signal peptidase S24, S26A and S26B [*S. suis* 89/1591].	ZP_00875273	
1B8	ssu0453	Sortase-like protein SrtE [*S. suis*]	BAB83972	(29)
Secreted proteases				
D1E9	ssu0457	Collagenase-Peptidase U32 [*S. suis*].	BAB83975	
Cell division / Replication				
1G5	ssu0002	DNA polymerase III, β-chain [*S. suis* 89/1591].	ZP_00875475	

2F4	ssu0432	Cell division protein FtsQ/DivIB [*S. suis* 89/1591]	ZP_00876117	(12)
Transport / Binding				
D1H3	ssu1787	Multidrug ABC transporter, ATP-binding protein [*B. cereus* E33L].	AAU18528	
D3G1	ssu1023	Putative permease [S. *suis* 89/1591]	ZP_00874974	
Unknown function				
D1G1	ssu0067	Protein of unknown function DUF925 [*Streptococcus suis* 89/1591].	ZP_00876271	
2F6	ssu1424	Hypothetical protein [*S. suis* 89/1591]	ZP_00875489	
D1H2	ssu0858	Protein of unknown function UPF0153 [*S. suis* 89/1591].	ZP_00875788	
D1A3	ssu1792	Conserved hypothetical protein [*S. suis* 89/1591].	ZP_00876058	

[1] Genes named after *S. suis* strain P 1/7 sequencing project nomenclature.

Article I Figures

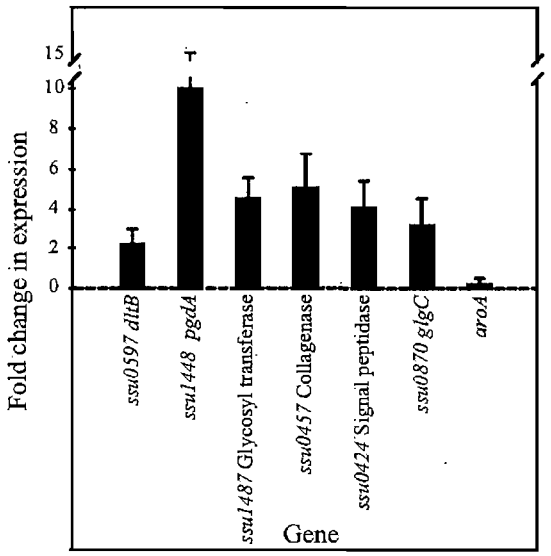

Figure 12. Article I, Figure 1. Fold change in expression of selected *S. suis* genes identified by SCOTS as measured by q-PCR upon interaction of the bacterium with PBMEC.
See the text for details.

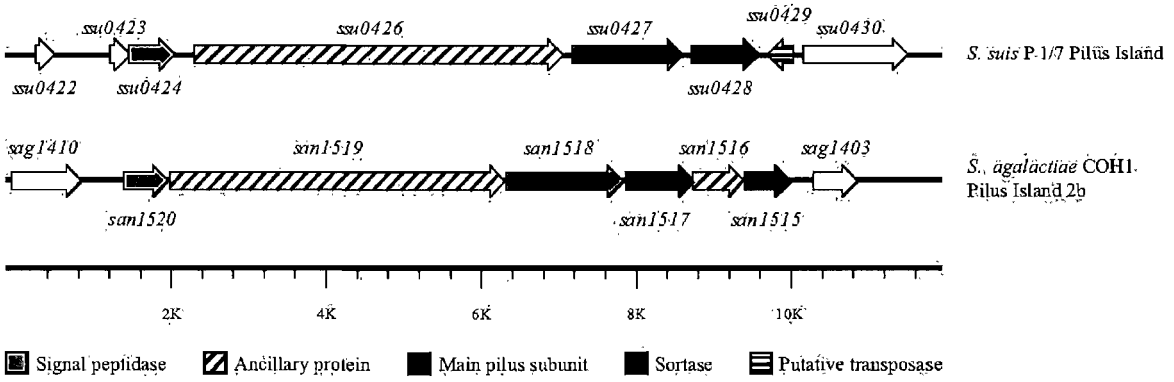

Figure 13. Article I, Figure 2. Putative pilus island in *S. suis* (upper panel) and its counterpart, pilus island 2b (PI-2b), found in *S. agalactiae* strain COH1 (lower panel).
In *S. suis*, the first gene of the locus is a LepB-type signal peptidase (identified by SCOTS and q-PCR to be upregulated upon interaction with PBMEC), which is followed downstream by a putative ancillary protein and a main pilus subunit. A previously undescribed class C sortase is the last gene of the island. The *S. agalactiae* PI-2b, which is organized in a similar way, comprises an additional ancillary pilus subunit and a second class C sortase (Genbank entry NZ_AAJR01000022).

ARTICLE II

"D-Alanylation of the Lipoteichoic Acid Contributes to the Virulence of *Streptococcus suis*"

Nahuel Fittipaldi, Tsutomu Sekizaki, Daisuke Takamatsu, Josée Harel, María de la Cruz Domínguez-Punaro, Sonja Von Aulock, Christian Draing, Corinne Marois, Marylène Kobisch and Marcelo Gottschalk.

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Details on the role of the candidate in the conception of the article: I substantially contributed to research design, performed research, analyzed data and wrote the paper.

D-Alanylation of the Lipoteichoic Acid Contributes to the Virulence of *Streptococcus suis*

Nahuel Fittipaldi[1], Tsutomu Sekizaki[2,3], Daisuke Takamatsu[2], Josée Harel[1], María de la Cruz Domínguez-Punaro[1], Sonja Von Aulock[4], Christian Draing[4], Corinne Marois[5], Marylène Kobisch[5] and Marcelo Gottschalk[1]*

[1]Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Qc, J2S 7C6, Canada; [2]Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan; [3]United Graduate School of Veterinary Sciences, Gifu University, 501-1193 Gifu, Japan; [4]Department of Biochemical Pharmacology, University of Konstanz, D-78457 Konstanz, Germany; [5]Agence Française de Sécurité Sanitaire des Aliments, Laboratoire d'Études et de Recherches Avicoles et Porcines, Unité de Mycoplasmologie-Bactériologie, 22440 Ploufragan, France.

Running title: LTA D-alanylation and *S. suis* virulence.

* Corresponding author. Phone (450) 773-8521 x 18374; Fax (450) 778-8108. E-mail: [redacted]

Abstract

We generated by allelic replacement a Δ*dltA* mutant in a virulent *Streptococcus suis* serotype 2 field strain and evaluated the contribution of lipoteichoic acid (LTA) D-alanylation to the virulence traits of this swine pathogen and zoonotic agent. Absence of LTA D-alanylation resulted in increased susceptibility to the action of cationic antimicrobial peptides. In addition, and in contrast to the wild type strain, the Δ*dltA* mutant was efficiently killed by porcine neutrophils and showed diminished adherence to and invasion of porcine brain microvascular endothelial cells. Finally, the Δ*dltA* mutant was attenuated in both the CD1 mouse and porcine models of infection, probably reflecting decreased ability to escape immune clearance mechanisms and impaired capacity to traverse across host barriers. The results of this study suggest that LTA D-alanylation is an important factor in *S. suis* virulence.

Introduction

Streptococcus suis is a major swine pathogen and a zoonotic agent responsible for, among other diseases, meningitis and septicemia (15). In swine, *S. suis* causes severe losses to the industry (15) while human *S. suis* infection is emerging as an important public health issue (13). Very recently, more than 200 cases of human *S. suis* infection were reported during an outbreak in China, 38 of which resulted in death (39). *S. suis* is considered the primary cause of adult meningitis in Vietnam (20) and human *S. suis* infection resulting in death or in severe post-infection sequelae has already been reported in different Asian and European countries as well as in New Zealand, Australia, Argentina, Canada and the United States (13). Among *S. suis* serotypes, serotype 2 is responsible for most cases of disease in both swine and humans, and almost all studies on virulence factors and pathogenesis of the infection have been carried out with this serotype (13, 15). Despite the increasing number of studies, our understanding of the pathogenesis of *S. suis* infection remains limited. The polysaccharide capsule is known to play a critical role in the pathogenesis of *S. suis* infection (15). It has been shown that unencapsulation of *S. suis* correlated with increased phagocytosis by porcine macrophages and killing by porcine neutrophils (4, 6, 29) and that it severely impaired virulence in a porcine model of infection (29). Recently, an isogenic mutant for a serum opacity-like factor was found to be highly attenuated in pigs (2). Other proposed putative virulence factors such as the suilysin, the extracellular protein factor, the muramidase-released protein and a fibronectin/fibrinogen-binding protein were found to be associated with and/or partially involved in, but not essential for, virulence (7, 15).

S. suis can affect the viability of porcine blood brain barrier (BBB)-forming cells such as porcine choroid plexus epithelial cells (CPEC) through necrotic and apoptotic mechanisms (34). It also can adhere to and invade *in vitro*-cultured porcine brain microvascular endothelial cells (porcine BMEC), another type of BBB-forming cells (35). The ability of *S. suis* to interact with these cells is thought to be important for attaining the central nervous system (CNS) and causing meningitis in swine (13). In a recent study (11), the selective capture of transcribed sequences (SCOTS) approach was used to elucidate genes that this pathogen preferentially upregulates upon its interactions with porcine BMEC. Among other genes, the study identified a gene member of a putative *S. suis dlt* operon (11). In all bacteria where this operon has been studied,

it has been found to be responsible for the incorporation of D-alanine residues into lipoteichoic acids (LTA), surface-associated amphiphilic molecules found in most Gram positive bacteria (23).

The cell wall of *S. suis* has been proposed as an important virulence factor. Several studies have shown that the cell wall or its purified components such as the LTA contribute to exacerbate the host inflammatory response to infection (13, 15). 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 (9). 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 (36). 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 (11). It is known from previous reports that D-alanylated LTA is important for the virulence of Gram-positive pathogens based on findings that it enables these organisms to modulate their surface charge, to regulate ligand binding and to control the electromechanical properties of the cell wall (23). In addition, formation of D-alanyl-LTA is required to resist the action of cationic antimicrobial peptides (CAMPs) (1, 17, 18, 26). The D-alanylation of *S. suis* LTA and its contribution to the pathogenesis of infection have yet to be documented. In this study, we demonstrate that *S. suis* D-alanylates its LTA and that this modification is important for the virulence traits of this pathogen.

Materials and methods

Bacterial strains, plasmids, media, culture conditions and chemicals.

Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, *S. suis* strains were grown in Todd-Hewitt (Becton Dickinson, Sparks, MD) broth (THB) or agar (THA) at 37°C under 5% CO_2. *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth or agar medium (Becton Dickinson) at 37°C. When needed, antibiotics (Sigma, Oakville, Ontario, Canada) were added to the culture media at the following concentrations: for *S. suis*, chloramphenicol (Cm) at 5 μg/ml and spectinomycin (Sp) at 100 μg/ml; for *E. coli*, kanamycin

(Km) and Sp at 50 μg/ml; Cm at 10 μg/ml. Unless otherwise indicated, all chemicals were purchased from Sigma.

DNA manipulations.

Restriction enzymes, DNA-modifying enzymes and Taq and Pwo DNA polymerases were purchased from GE Healthcare (Piscataway, NJ, USA) or Takara Bio (Otsu, Shiga, Japan) and used according to the manufacturers' recommendations. *S. suis* genomic DNA was prepared by the guanidium thiocyanate method (24). Mini-preparations of recombinant plasmids from *E. coli* and transformation of *E. coli* were performed by standard procedures (27). Southern hybridizations were performed by the procedures described previously (28), except that hybridization was carried out at 68°C. For preparation of probes, DNA fragments were labelled with digoxigenin (DIG) using the DIG-PCR labeling mixture (Roche Diagnostics, Laval, Qc, Canada) according to the manufacturer's instructions. Oligonucleotide primers were from Invitrogen (Burlington, Ontario, Canada).

Allelic replacement.

(i) Construction of the knockout vector. DNA fragments corresponding to regions upstream and downstream the *dltA* gene (Fig. 1A) were amplified from genomic DNA of *S. suis* strain 31533 by PCR using the primer sets 2872F plus 3765R (left arm) and 5250F plus 5809R (right arm). A Sp resistance cassette (gene *aad9*) was amplified from plasmid pSmall with primers specF3 and specR. All three primers sets introduce unique restriction sites (Table 2). PCR amplicons were digested using the appropriate restriction enzymes, and sequentially ligated in the order left arm-Sp cassette-right arm using T4 DNA ligase. The resulting fragment was amplified by PCR using primers 2872F and 5809R, cloned into vector pCR4 (TOPO TA PCR cloning kit; Invitrogen), excised with HindIII and BamHI and recloned into the HindIII and BamHI sites of the temperature sensitive *S. suis-E. coli* shuttle vector pSET5s, which carries the gene *cat* conferring Cm resistance (32), giving rise to knockout vector p5ΔdltA (Fig. 1B). (ii) Generation of *S. suis ΔdltA*. Procedures for selection of mutants by allelic exchange via double crossover have been described previously (32). Briefly, *S. suis* strain 31533 was transformed with p5ΔdltA by electroporation as previously described (31). The cells were grown at 28°C in the presence of Cm and Sp selection. Bacteria at mid-logarithmic growth phase were diluted with THB containing Sp

and grown at 28°C to early logarithmic phase. The cultures were then shifted to 37°C and incubated for 4 h. Subsequently, the cells were spread onto THA agar containing Sp and incubated at 28°C. Temperature-resistant, Sp-resistant colonies were screened for loss of vector-mediated Cm resistance to detect putative mutants which had exchanged their wild-type allele for a genetic segment containing the *aad9* gene as a consequence of homologous recombination via a double crossover. Allelic replacement in candidate clones was verified by PCR and Southern hybridization, which confirmed the expected genotype (data not shown).

Transmission electron microscopy.

Transmission electron microscopy was performed as previously described (12). Briefly, overnight (ON) cultures of *S. suis* wild type (WT) or mutant Δ*dltA* strains were mixed with rabbit anti-*S. suis* serotype 2 polyclonal serum and incubated at room temperature for 1 h. Cells were then fixed in cacodylate buffer (0.1M cacodylate, 5% glutaraldehyde, 0.15% ruthenium red, pH 7.2) for 2 h. After fixation, cells were immobilized in 4% agar, washed in cacodylate buffer and post-fixed ON at 4°C in 2% osmium tetraoxyde. Samples were dehydrated in graded series of ethanol and embedded in Spurr low-viscosity resin. Thin sections were post-stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Model 420, Philips Electronics, The Netherlands).

Determination of LTA D-alanine content.

S. suis WT and Δ*dltA* mutant strains were cultured in tryptic soy broth (Becton Dickinson) containing beef extract (5 g/l) and glucose (8 g/l) at 37°C for 18 h with shaking. After incubation, bacteria were harvested by centrifugation at 4225 x g for 20 min. Integrity of bacteria and potential contamination by Gram-negative species were checked by Gram staining and microscopy. LTA were prepared by butanol extraction (which preserves the integrity of the D-alanine substitutions) and hydrophobic interaction chromatography as previously described (22). Nuclear magnetic resonance (NMR) spectra from LTA were recorded on a Bruker Avance DRX 600 spectrometer (Bruker BioSpin, Ettlingen, Germany) equipped with an inverse TXI-H/C/N triple resonance probe at 300 K using 3 mm Bruker Match sample tubes. Spectra were measured in D_2O using sodium 3-trimethylsilyl- 3,3,2,2-tetradeutero-propanoate as an internal standard for ^1H NMR (δ_H 0.00 ppm).

Antimicrobial peptides sensitivity.

Assays were carried out in sterile 96-well microtiter plates. Logarithmic-phase *S. suis* cells were adjusted to approximately 10^4 CFU/ml in 100 µl THB containing one of the following antimicrobial compounds in serial dilutions: Colistin (0 to 200 µg/ml), polymyxin B (0 to 300 µg/ml) and magainin II (0 to 45 µg/ml). Plates were incubated for 24 h at 37°C. The MIC was defined as the lowest antimicrobial concentration yielding no detectable bacterial growth by $OD_{600\ nm}$ measurement.

Killing by porcine neutrophils.

Experiments were carried out as described previously (4). Briefly, blood samples were collected by venous puncture from high health status pigs which tested negative by ELISA (19) for *S. suis* serotype 2. Cell populations were separated by Ficoll-Hypaque (GE Healthcare) density gradient centrifugation and neutrophils isolated by sedimentation in 6% dextran. Contaminating erythrocytes were removed by lysis with 0.83% ammonium chloride. Neutrophils were resuspended at a final concentration of 5×10^6 cells/ml in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated porcine serum. Bacteria (WT or Δ*dltA* mutant strains at approx. 1×10^4 CFU/ml) were opsonized with complete normal porcine serum for 30 min at 37°C and then mixed in microtubes with neutrophils at 5×10^6 cells/ml. The mixture was incubated for 90 min at 37°C under 5% CO_2. Under these conditions bacteria are not toxic to neutrophils (4). After incubation cells were lysed with sterile water and viable bacterial counts on THA were performed.

Experimental infections.

All experiments involving animals were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care. (i) Pigs: A total of 20, 4-week-old, second-generation of caesarean-derived pigs were used in this study. Strict biosecurity measures were implemented to avoid undesirable contamination of the pigs; these included an air filtration system and airlocks for each unit. Pigs were divided in 3 groups. Six out of 7 animals in groups 1 and 2 were inoculated by intravenous injection of 10^8 CFU of the *S. suis* WT 31533 or mutant Δ*dltA* strains, respectively. The remaining animal in both the WT and Δ*dltA* mutant groups was not inoculated although it was housed with inoculated animals and served as sentinel. Group 3

(N=6) were sham-inoculated animals. Clinical signs and presence of *S. suis* in blood were monitored during the trial. Surviving animals in all 3 groups were sacrificed 7 days pi and examined for pathological lesions. Bacteriological isolation from different organs (liver, spleen, lungs, heart and articulations) was performed as described below for mice. (ii) CD1 mice: A recently described murine model for *S. suis* infection was used (8). A total of 60 female CD1 mice aged 6 weeks (Charles River laboratories, Wilmington, MA) were used for virulence assessment. At day 0, animals were divided in 4 groups of 15 mice. Group 1 was inoculated by intraperitoneal injection of 1 ml of *S. suis* strain 31533 suspension at 5 x 10^7 CFU/ml, while group 2 received the same dose of mutant strain *ΔdltA*. Groups 3 and 4 received 1 ml of a 5 x 10^6 CFU/ml suspension of the WT and the mutant strains, respectively, using the same route of inoculation. Mice were monitored 3 times/day for 10 days for clinical signs and assigned clinical scores as previously described (8). Blood samples (5 µl) were collected daily (from the tail vein) and at euthanasia (by cardiac puncture) and used to evaluate bacterial load by plating onto sheep blood agar plates. Isolated tiny α-hemolytic colonies were counted and assigned to *S. suis* by serotyping as previously described (16). Surviving animals in both groups were sacrificed at day 10 and macroscopic examination was performed. Bacterial colonization of the liver, spleen and brain of infected animals was also evaluated. Briefly, small pieces of these organs weighing 0.5 g were trimmed, placed in 500 µl of PBS and homogenized. Thereafter, 50 µl of the suspensions were plated as described above. In addition, an enrichment of the samples was carried out by inoculation of 300 µl of homogenized organ samples or 100 µl of blood into THB, followed by ON incubation at 37°C and subsequent dilution and plating onto sheep blood agar plates as described above.

Adherence to and invasion of porcine BMEC.

The porcine BMEC cell line PBMEC/C1-2 (33) was grown in Primaria 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) using IF culture medium (a mixture of 1:1 Iscove's modified Dulbecco's and Ham's F-12 media, Invitrogen) supplemented as previously described (35). *S. suis* was grown in THB for 16 h at 37°C, harvested by centrifugation, washed twice in phosphate-buffered saline (PBS), pH 7.3, and resuspended in fresh IF culture medium. The invasion assays were performed as described previously (35). Briefly, confluent monolayers of porcine BMEC at 10^5 cells/well were infected with 1-ml aliquots of bacterial suspensions at 10^5 CFU/ml (multiplicity of infection (MOI) of 1). The plates were centrifuged at 800 x *g* for 10

min and incubated for 2 h at 37°C with 5% CO_2. The monolayers were then washed twice with PBS. A 1 ml volume of cell culture medium containing 100 µg/ml of gentamicin and 5 µg/ml of penicillin G was added to each well, and incubation continued for 1 h. After incubation, monolayers were washed 3 times with PBS, trypsinized and disrupted by repeated pipetting. Serial dilutions of the cell lysates were plated onto THA and incubated ON at 37°C. To confirm that 100% of the extracellular bacteria were killed after the antibiotic treatment, a 100-µl sample of the last PBS wash was plated onto THA (results not shown). Adherence assays were performed essentially as described for invasion, but neither antibiotic treatment nor extended incubation was performed. After 2 h incubation, cells were vigorously washed 5 times with PBS, trypsinized, disrupted, and serial dilutions of the cell lysates were plated as described above.

Results and Discussion

The *dlt* operon is responsible for LTA D-alanylation in *S. suis*.

The genetic organization of the *S. suis dlt* operon is depicted in Fig. 1A. Sequence comparison at The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), as well as previous reports (17, 18, 25), showed that the *S. suis dlt* operon is organized in a fashion similar to that of all *dlt* operons reported in pathogenic streptococci so far, with the exception of *S. agalactiae*, which also includes 2 regulatory genes upstream of the *dltA* gene (25). Accordingly, the deduced proteins showed a high degree of similarity to streptococcal Dlt proteins (data not shown). To assess the contribution of the *dlt* operon to LTA D-alanylation, we constructed by allelic replacement a Δ*dltA* mutant strain and analyzed the content of D-alanine in purified LTA of the WT and Δ*dltA* mutant strains by NMR. Fig. 2 shows the NMR spectra for LTA of the two strains. Both LTA showed the expected peaks for fatty acids (0.85 and 1.3 ppm) and sugars (3.5 to 4.5 ppm). However, peaks for D-alanine (1.65, 4.3 and 5.4 ppm) were absent in the Δ*dltA* mutant spectrum, suggesting that the LTA of the mutant is devoid of this amino acid substitution. *In vitro* growth of the Δ*dltA* mutant was comparable to that of the WT strain (Fig. 3A) and no other major phenotypic changes were observed. In contrast to previous reports on *S. agalactiae* and *Streptococcus pyogenes* Δ*dltA* mutants, which were either poorly separated or multiseptated in the stationary phase of growth (18, 26), the *S. suis* Δ*dltA* mutant was encapsulated, well separated and presented normal septation (Fig. 3B).

S. suis LTA D-alanylation contributes to antimicrobial peptide resistance and decreases susceptibility to neutrophil killing.

CAMPs kill bacteria by forming pores in the cytoplasmic membrane (30). Introduction of positively charged D-alanine residues into the LTA would reduce the global negative charge of the *S. suis* envelope, thus providing the bacterium with a physical mechanism of resistance against the action of CAMPs (23). To assess this hypothesis, we performed an evaluation of the sensitivity of the WT and Δ*dltA* mutant to selected CAMPs. The *S. suis* Δ*dltA* mutant was more sensitive than the WT strain against the bacteria-derived cationic peptide polymyxin B and colistin and the frog-derived peptide magainin II (Table 3). These results were in agreement with previous reports of inactivation of the *dltA* gene in streptococcal species (17, 18, 26) and indicate that D-alanylation of LTA is an important component of the intrinsic resistance of *S. suis* to CAMPs killing. On the other hand, the WT and *dltA* mutant strains were equivalent in their susceptibility to the antibiotics gentamicin and penicillin G and to lysozyme (data not shown). Functional homologues of the CAMPs tested in this study are secreted by neutrophils both into the phagosome as well as extracellularly. (21). When we compared killing of WT and Δ*dltA* mutant strains by purified porcine neutrophils, in agreement with a previous study (4), the WT strain avoided killing by neutrophils when opsonized with normal complete porcine sera. On the other hand, 20% of the Δ*dltA* mutant bacteria were killed by neutrophils (Fig. 4). This level of killing was similar to that of the unencapsulated mutant strain BD102, despite the fact that the Δ*dltA* mutant does not have altered capsule expression (Fig. 4). This was surprising, since encapsulated WT *S. suis* has been shown to prevent phagocytosis by porcine neutrophils (unless opsonized by specific antibodies) (4). However, it is known that neutrophils are also able to destroy infecting microorganisms in the absence of phagocytosis in the so-called neutrophil extracellular traps (NETs) (3). Interestingly, it has recently been shown in *Streptococcus pneumoniae* that absence of LTA D-alanylation results in enhanced extracellular killing in NETs by neutrophils but not in an increased phagocytosis of this organism by these polymorphonuclear cells (37). Although our killing assay is not able to discriminate between intra and extracellular killing, taking all these findings together, it might be proposed that the encapsulated *S. suis* Δ*dltA* mutant is killed by porcine neutrophils extracellularly, perhaps after being trapped in NETs. In addition, we speculate that the enhanced killing of the *S. suis* Δ*dltA* mutant might be the consequence of the absence of LTA D-alanylation, which results in an

increased susceptibility to CAMPs released by neutrophils. Further experiments are needed to evaluate this hypothesis.

Virulence of the Δ*dltA* mutant is attenuated in pigs.

Several Δ*dltA* mutants in different Gram positive pathogens have been reported and almost all of these mutants were highly susceptible to CAMPs and killing by neutrophils and/or macrophages (1, 5, 10, 14, 17, 18, 26, 37, 38). However, only a limited number of studies have analyzed *in vivo* the contribution of LTA D-alanylation to the virulence of these pathogens. In these cases, virulence of the tested Δ*dltA* mutants greatly varied between bacterial species preventing the drawing of conclusions regarding the contribution of LTA D-alanylation to virulence traits of pathogens from previous studies (1, 26, 37, 38). Finally, for various valid reasons, previous studies on the virulence of Δ*dltA* mutants in Gram positive species have used surrogated models of infection instead of the natural hosts (1, 26, 37, 38). *S. suis* shares certain characteristics with pathogens for which Δ*dltA* mutants have been described. However, its pathogenesis of infection is essentially different (15). In this study, we have for the first time evaluated the virulence of a Gram positive Δ*dltA* mutant in the context of its natural host by intravenous inoculation of pigs. Animals in the sham-inoculated group did not present any clinical signs during the entire duration of the trial. In contrast, severe clinical signs were recorded in 5 out of the 6 animals inoculated with the WT strain during the first 4 days of the trial. These 5 pigs died or were sacrificed for ethical reasons at day 2 pi (3 animals) and at day 4 pi (2 pigs). The remaining inoculated animal and the sentinel pig in this group survived until the end of the trial (Fig. 5). Animals infected with the Δ*dltA* mutant presented, on average, less severe clinical signs during the 4 first days pi. However, 2 animals died and an additional was euthanized by ethical reasons in this group (Fig. 5). Nevertheless, the remaining inoculated animals noticeably recovered starting at day 4 pi, and, along with the sentinel pig in the Δ*dltA* group, survived until the end of the trial. Hyperthermia (> 40.5°C) was observed in all pigs infected with either WT or Δ*dltA* strain at 24 h pi. Temperatures returned to normal values after day 4 in both groups. However, in the WT group, the sentinel pig developed hyperthermia from day 6 pi. *S. suis* serotype 2 could be isolated from the blood of all inoculated pigs in both groups and the sentinel animal in the WT group. Pigs in the latter group had higher bacterial counts (as high as 1×10^{10} CFU/ml in some cases) than those infected with the Δ*dltA* mutant (average of 1×10^{8} CFU/ml) during the first 4 days pi. Similar to the blood, bacterial titers in organs were

slightly lower in pigs inoculated with the Δ*dltA* mutant compared with animals inoculated with the WT strain. However, examination at necropsy did not find major differences between the WT and Δ*dltA* mutant groups regarding damage to tissues or organs. At euthanasia, macroscopic lesions typical of *S. suis* infection were found in most animals infected with the WT or Δ*dltA* strains, especially at the pleura, pericardium and peritoneum. Fibrin deposits were observed in the liver and spleen of most animals in both groups. Pneumonia and fibrinal pleurisy were also observed in some animals. Additionally, the meninges showed inflammation consistent with meningitis. Lameness was observed in all pigs infected with the WT or Δ*dltA* strain. At necropsy, articulations showed inflammation, with fibrin deposits and excess of synovial liquid. Results from the experimental infection showed that the Δ*dltA* mutant is attenuated in the pig and suggest that LTA D-alanylation provides an advantage to the WT strain. However, this conclusion is mitigated by the facts that dissemination of the bacterium was not prevented and mortality was observed among animals inoculated with the Δ*dltA* mutant. Since clearance of the mutant from circulation might primarily rely on neutrophil activity, the high dose used to inoculate the animals may explain, at least in part, the mortality observed. Indeed, it has been proposed that suilysin may affect complement activity and suilysin-producing *S. suis* strains, such as the WT and mutant strains used in this study, have been shown to be toxic to neutrophils at high titers (4). In addition, since CAMPs activity primarily occurs at mucosal surfaces, the extremely aggressive intravenous route of administration may have also influenced the clinical onset observed in pigs.

Absence of LTA D-alanylation impairs *S. suis* virulence in mice.

To better evaluate the attenuation of the *S. suis* Δ*dltA* mutant observed in the pig we performed additional *in vivo* trials using the CD1 mouse model of infection that uses the intraperitoneal route of inoculation (8). We performed two different trials at high and intermediate doses of infection. At the high dose (5×10^7 CFU per animal) most mice in both the WT and Δ*dltA* mutant groups presented severe clinical signs associated with septicemia, such as depression, swollen eyes, weakness and prostration during the first 72 h pi. At this dose we did not find a clear reduction in the ability of the Δ*dltA* mutant to successfully initiate infection and induce septicemia in mice. In fact, several mice died from septicemia in both groups during the first 3 days of the trial (Fig. 6A). *S. suis* was isolated at high titers (> 1×10^7 CFU/ml) from blood samples and organs such as the liver and spleen of septicemic animals (> 1×10^7 CFU/0.5 g of

tissue in some animals). From day 5 pi, some mice in both WT and Δ*dltA* groups developed clinical signs associated with *S. suis* meningitis in the mouse (8), such as hyperexcitation, episthotonus, opisthotonus, bending of the head and walking in circles. It has been proposed that maintaining a high level of bacteremia is essential for CNS disease to appear at later stages of the infection (13). Interestingly, the number of meningitis-presenting mice was lower in the Δ*dltA* group (N=1) than in the WT group (N=6), and this observation was consistent with the reduction in the bacterial load in blood among animals inoculated with the Δ*dltA* mutant compared to those having received the WT strain (data not shown). Therefore, we performed a second trial in the mouse using an intermediate dose of infection (5 x 10^6 CFU per animal) in order to avoid development of septicemia. Mice in both groups presented moderate clinical signs during the first 72 h pi, but no animals died from septicemia in either group. However, from day 7 pi, several mice in the WT group developed clinical signs associated with meningitis. *S. suis* was isolated from the brain of these animals at high titers (>1 x 10^6 CFU/0.5 g of tissue). In strong contrast, no clinical signs of meningitis were observed in the Δ*dltA* group nor was *S. suis* isolated from the brain of any animal infected with the Δ*dltA* mutant. Significant differences in the mortality rate were noted between mice inoculated with the WT and those with the Δ*dltA* mutant strain (Kaplan-Meyer, $P < 0.05$) at the intermediate dose of infection (Fig. 6B).

***S. suis* LTA D-alanylation promotes adherence to and invasion of porcine BMEC.**

Experimental infection of mice at the intermediate dose of infection clearly demonstrated that the Δ*dltA* mutant is less fit to induce CNS disease. A recent study of *S. suis* meningitis in the mouse showed that cells lining the choroid plexus and the brain endothelium are potential entry sites for this pathogen into the CNS (8). In addition, previous studies demonstrated the ability of *S. suis* to adhere to and invade immortalized porcine BMEC (35, 36). Recently, it has been shown that expression of the *dlt* operon is upregulated upon interaction of *S. suis* with porcine BMEC (11). Therefore, to assess the contribution of the LTA D-alanyl modification to adherence to and invasion of porcine BMEC, we compared the interactions of WT and Δ*dltA* mutant strains with cultured monolayers of these cells. After 2 h-incubation of *S. suis* with porcine BMEC at a MOI of 1, followed by vigorous washing, we found a marked decrease in the total number of cell-associated Δ*dltA* mutant bacteria compared with the WT parent strain (Fig. 7). Using antibiotic protection to quantify bacteria which had invaded the intracellular compartment, a similar reduction in internalization of the Δ*dltA* mutant was

observed (Fig. 7). Therefore, LTA D-alanylation itself plays a role in facilitating *S. suis* adherence to and invasion of porcine BMEC and we speculate that this occurs, mainly, through cell envelope charge stabilization that allows efficient display of proteinaceous adhesins and/or invasins (23). Porcine BMEC are one of the main cellular types forming the porcine BBB, a structure that successful meningitis-causing pathogens must cross in order to develop meningitis. Interestingly, a previous report proposed that the diminished resistance to killing by leucocytes was responsible for the impairment in its ability to induce meningitis in the mouse of a *S. agalactiae* Δ*dltA* mutant (26). Taking together our results with porcine BMEC and the observed outcome of the experimental infections in both the murine and porcine models of infection, we speculate that in addition to failure of the Δ*dltA* mutant to maintain a high level of bacteremia, impaired interaction with BMEC are also responsible for the reduced ability of the Δ*dltA* mutant to induce meningitis.

In summary, *S. suis* LTA D-alanylation mediated by the *dlt* operon contributes phenotypically to resistance to CAMPs, likely through an increased net positive surface charge. It also enhances the resistance of *S. suis* to neutrophil killing as well as its capacity for adherence to and invasion of porcine BMEC. In addition, LTA D-alanylation contributes to *S. suis* virulence in both the murine and the porcine models of infection, probably through interference with innate immune clearance mechanisms and by facilitating penetration of host barriers. The results of this study strongly suggest that LTA D-alanylation is an important virulence factor of this swine pathogen and zoonotic agent.

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Article II Tables

Table IV. Article II, Table 1. Bacterial strains and plasmids used in this study

	Relevant characteristics	Source
Bacterial strains		
E. coli Top 10	General strain for cloning. F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
S. suis 31533	Serotype 2 field strain. Highly virulent	(35)
S. suis Δ*dltA*	Δ*dltA* mutant strain derived from strain 31533	This work
S. suis BD102	Unencapsulated mutant strain derived from strain 31533	(12)
Plasmids		
pCR4	*E. coli* vector for cloning of PCR fragments	Invitrogen
pSmall	*E. coli-S. suis* shuttle vector. Sp^R. Source of *aad9*	Willson, P (unpublished results)
pSET5s	Temperature sensitive suicide vector for *S. suis* mutagenesis. Cm^R (*cat*)	(32)
p5ΔpgdA	pSET5s carrying the construction for allelic exchange	This work

Table V. Article II, Table 2. Oligonucleotide primers used in this study. Restriction sites are in bold

Primer name	Sequence (5′ – 3′)	Restriction site
2872F	GCAGTTACCTCTAAGCTTGCGACAACGG	HindIII
3765R	CTGCTAATCATTTGGATCCTCTCCTC	BamHI
5250F	CTTCCTTTGACTGCAGATGGGAAGATT	PstI
5809R	CGTCTATAAGGATCCATAGGG	BamHI
specF3	GCCAATGAGATCTATAAATAAAC	BglII
specR	AAAGTGTTTCCTGCAGTTTTTCAA	PstI

Table VI. Article II, Table 3. Sensitivity of the *S. suis* WT and Δ*dltA* mutant strains to the action of selected antimicrobial peptides.

		MICs (μg/ml)	
Peptide (origin)	Net charge	31533 (WT)	Δ*dltA*
Colistin (*Bacillus colistinus)*	+5	50	25
Polymyxin B (*Bacillus polymyxa*)	+5	75	18.75
Magainin II (claw frog skin)	+4	45	5.6

Article II Figures

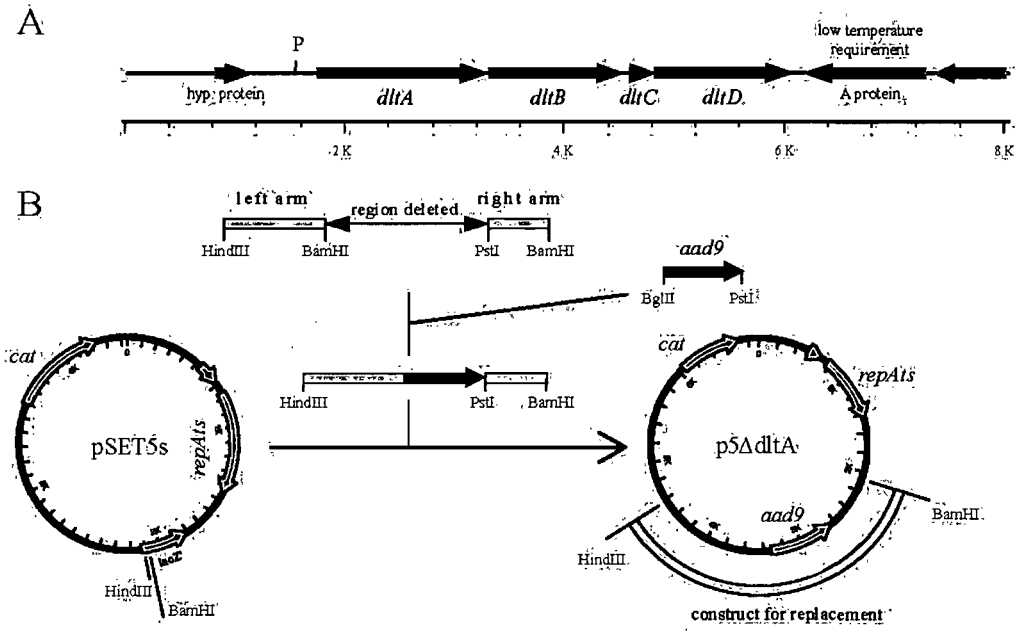

Figure 14. Article II, Figure 1. Genetic organization of the *S. suis dlt* operon and strategy for the construction of the Δ*dltA* mutant.

A) Genetic organization of the *S. suis dlt* operon as determined by sequencing of the region in strain 31533 and comparison with data from sequenced strain P1/7 available at the Sanger Institute (http://www.sanger.ac.uk/Projects/S_suis/). The *S. suis dlt* operon comprises 4 genes, *dltABCD*, and extends for 4340 bp. A putative strong promoter (indicated by P) was predicted upstream of the *dltA* gene. The operon comprises 4 genes, *dltA* (1563 bp), *dltB* (1242 bp), *dltC* (240 bp) and *dltD* (1266 bp). A putative strong promoter was predicted 228 bp upstream of the start codon for *dltA* using the software package Softberry BProm (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). B) Strategy followed in this study to construct the knock-out vector used to generate the Δ*dltA* mutant. See Materials and Methods for details.

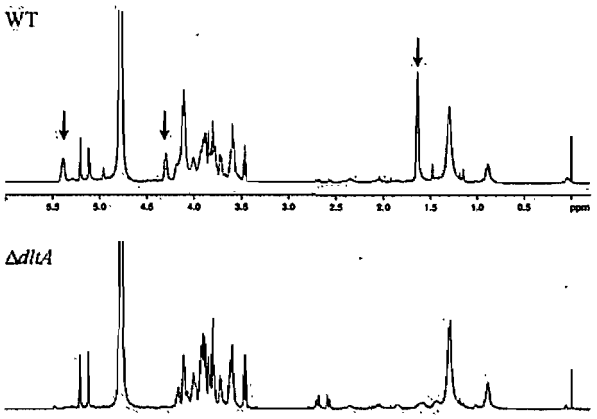

Figure 15. Article II, Figure 2. Nuclear magnetic resonance spectra of WT (upper panel) and Δ*dltA* mutant (bottom panel) LTA analysis.

The arrows show the peaks for D-alanine residues in the WT strain spectrum. These peaks are missing in the Δ*dltA* mutant spectra. No other differences between LTA of the two strains were found.

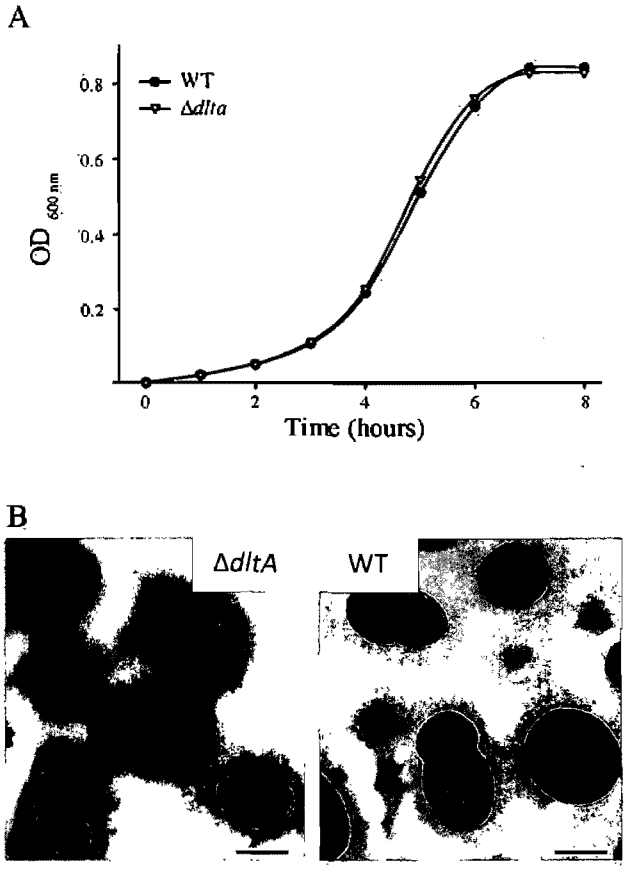

Figure 16. Article II, Figure 3. Growth curves and morphology of the WT and Δ*dltA* mutant strains.

A) Growth curves of *S. suis* WT and Δ*dltA* mutant strains. The Δ*dltA* mutant grew similarly as the WT parent strain under normal laboratory conditions. B) Morphology of the Δ*dltA* mutant (left) and the WT (right) strains. Transmission electron microscopy showed that both strains were well separated, presented normal septation and were surrounded by a thick polysaccharide capsule. Bar = 0.5 µm.

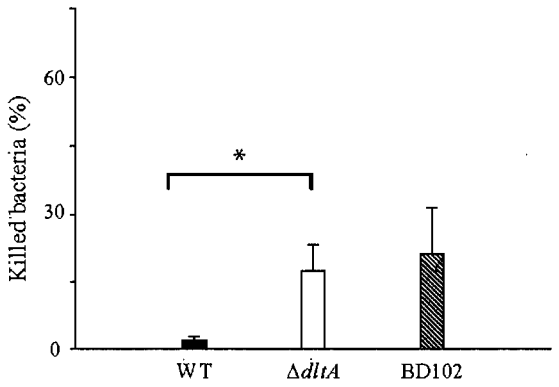

Figure 17. Article II, Figure 4. Percentage of bacteria killed after 90 min incubation with porcine neutrophils.

The different strains were opsonized with complete porcine sera before incubation. The level of killing of the Δ*dltA* mutant was similar to that observed for the unencapsulated mutant BD102 and significantly higher than that of the WT strain. Data are from at least 3 independent experiments. Error bars show standard deviation. Asterisks indicate significant differences, t-test, $P<0.05$.

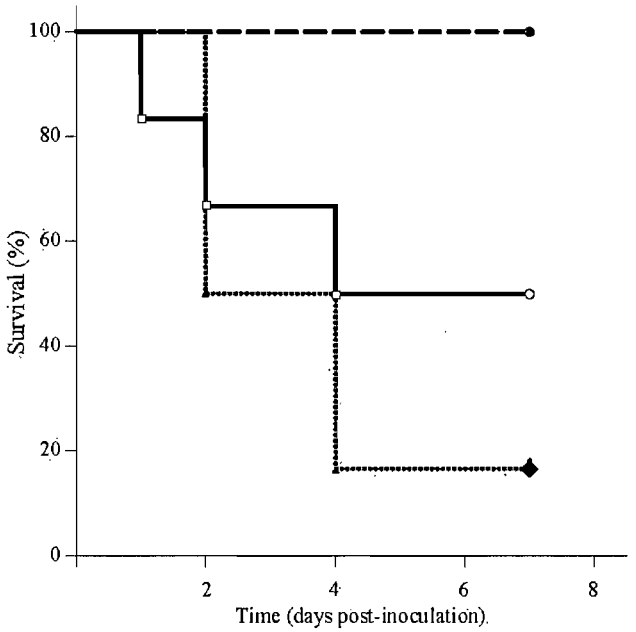

Figure 18. Article II, Figure 5. Survival of pigs inoculated with the WT (dotted line) or the Δ*dltA* mutant (solid line) strains and pigs that were sham-inoculated (dashed line).

All the sham-inoculated animals survived the trial. The survival rate of pigs in the Δ*dltA* mutant group was 50%, while in the WT group most animals died from septicemia in the first days of the trial (survival rate of 17%). The sentinel animals were not considered in this analysis. See text for details.

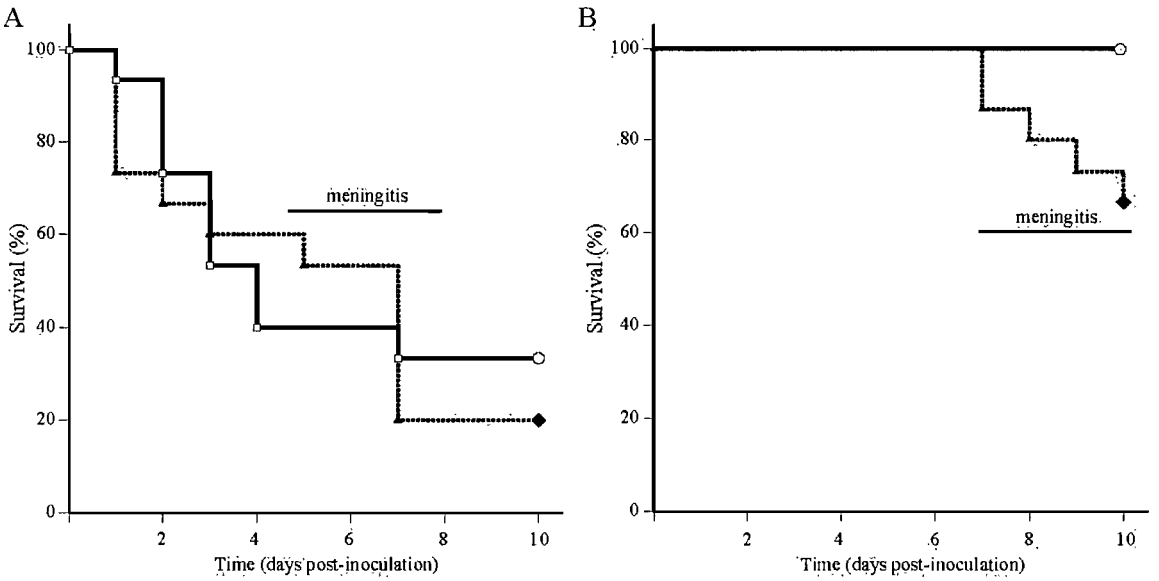

Figure 19. Article II, Figure 6. Survival of mice inoculated with the WT (dotted line) or the Δ*dltA* mutant (solid line) strains.

A)No significant differences in survival were observed between groups at the high dose of inoculation. However, fewer animals in the mutant group died from meningitis. (B) At the intermediate dose of inoculation all mice in the Δ*dltA* mutant group survived, while 35% of the WT group died from meningitis. Significant differences in survival, Kaplan-Meyer, $P<0.05$.

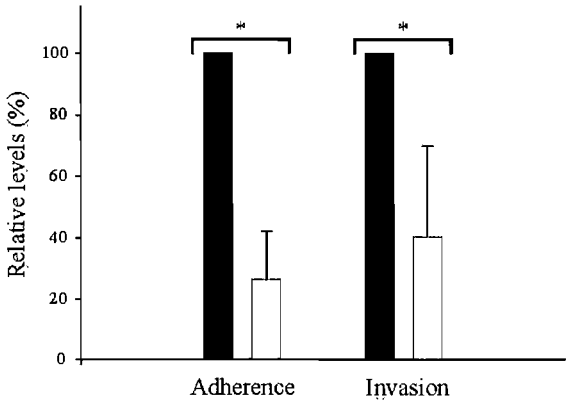

Figure 20. Article II, Figure 7. Interactions of the Δ*dltA* mutant and the WT strains with porcine BMEC.

The Δ*dltA* mutant showed reduced levels of adherence to and invasion of porcine BMEC. Data for the WT strain has been normalized to 100%. Data are from at least 4 independent experiments. Error bars show standard deviation. Asterisks indicate significant differences, t-test, $P<0.05$.

ARTICLE III

"Significant contribution of the pgdA gene to the virulence of Streptococcus suis"

Nahuel Fittipaldi, Tsutomu Sekizaki, Daisuke Takamatsu, María de la Cruz Domínguez-Punaro, Josée Harel, Nhat Khai Bui, Waldemar Vollmer and Marcelo Gottschalk

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Author contributions: NF and MG designed research; NF, MCDP and NKB performed research, TS, DT and WV contributed new reagents/analytic tools; NF, JH, WV and MG analyzed data; NF wrote the manuscript and MG and WV revised the manuscript.

Details on the role of the candidate in the conception of the article: I substantially contributed to research design, performed research, analyzed data and wrote the paper.

Significant contribution of the *pgdA* gene to the virulence of *Streptococcus suis*

Nahuel Fittipaldi[1], Tsutomu Sekizaki[2,3]†, Daisuke Takamatsu[2], María de la Cruz Domínguez-Punaro[1], Josée Harel[1], Nhat Khai Bui[4], Waldemar Vollmer[4] and Marcelo Gottschalk[1]*

[1]Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Qc, J2S 7C6, Canada. [2]Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan, [3]United Graduate School of Veterinary Sciences, Gifu University, 501-1193 Gifu, Japan, [4]Institute for Cell and Molecular Biosciences, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, United Kingdom.

Running title: Muropeptide N-deacetylation and *S. suis* virulence.

Key words: *Streptococcus suis*, peptidoglycan, *pgdA*, N-deacetylation, virulence, pig.

* For correspondence. E-mail: [redacted]; Tel. (+1) 450-773-8521, ext. 18374; Fax (+1) 450-778-8108.

† Present Address: Research Center for Food Safety, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan.

Summary

Streptococcus suis is a major swine pathogen and emerging zoonotic agent. In this study we have determined the muropeptide composition of *S. suis* peptidoglycan (PG) and found, among other modifications, N-deacetylated compounds. Comparison with an isogenic mutant showed that the product of the *pgdA* gene is responsible for this specific modification which occurred in very low amounts. Low level of PG N-deacetylation correlated with absence of significant lysozyme resistance when wild type *S. suis* was grown *in vitro*. On the other hand, expression of the *pgdA* gene was increased upon interaction of the bacterium with neutrophils *in vitro* as well as *in vivo* in experimentally inoculated mice, suggesting that *S. suis* may enhance PG N-deacetylation under these conditions. Evaluation of the Δ*pgdA* mutant in both the CD1 murine and the porcine models of infection revealed a significant contribution of the *pgdA* gene to the virulence traits of *S. suis*. Reflecting a severe impairment in its ability to persist in blood and decreased ability to escape immune clearance mechanisms mediated by neutrophils, the Δ*pgdA* mutant was severely attenuated in both models. The results of this study suggest that modification of PG by N-deacetylation is an important factor in *S. suis* virulence.

Introduction

Streptococcus suis is a major swine pathogen responsible for severe economic losses to the porcine industry (Higgins & Gottschalk, 2005). It is also the causative agent of serious infections in humans, especially in people in close contact with swine or pork by-products (Gottschalk *et al.*, 2007). In both swine and humans the main clinical manifestations of *S. suis* are meningitis and septicaemia. While *S. suis* outbreaks in swine are common and sometimes devastating, most reports in humans describe sporadic cases resulting in either death or severe post-infection sequelae (Gottschalk *et al.*, 2007). In recent times, however, *S. suis* has strongly emerged as an important public health issue in some Asian countries. For instance, it has very recently been shown that it is the primary cause of adult meningitis in Vietnam (Mai *et al.*, 2008). Moreover, during an episode in China, more than 200 human *S. suis* cases, 39 of which resulting in death, were reported in a single outbreak (Yu *et al.*, 2006). Most cases of disease in both swine and humans are caused by *S. suis* serotype 2 and, therefore, almost all studies on virulence factors and pathogenesis of the infection have been carried out with this serotype (Higgins & Gottschalk, 2005). However, the current understanding of the *S. suis* pathogenesis of infection remains limited. It has been shown that the polysaccharide capsule is essential for the virulence of the bacterium (Chabot-Roy *et al.*, 2006, Charland *et al.*, 1998, Smith *et al.*, 1999). Recently, an isogenic mutant for a serum opacity-like factor was found to be attenuated in pigs (Baums *et al.*, 2006). Several other factors, such as a heamolysin (suilysin), the so-called extracellular protein factor and muramidase-released protein, as well as a fibronectin/fibrinogen-binding protein, have been shown to be linked to, but not essential for, the virulence of *S. suis* (de Greeff *et al.*, 2002, Higgins & Gottschalk, 2005).

Cell wall components of *S. suis* have also been proposed to be important virulence factors and several studies have shown that they contribute to exacerbate the host inflammatory response to infection (Gottschalk et al., 2007, Higgins & Gottschalk, 2005). The main cell wall component of Gram-positive bacteria is peptidoglycan (PG), which ensures the stability and rigidity of the cell wall. PG consists of glycan strands made of alternating β-1,4-linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) which are cross-linked by short peptide chains (Vollmer *et al.*, 2008a). This rather invariant structure is exploited by the host for both recognition of bacteria, through the nucleotide-binding oligomerisation domain (Nod)1 and Nod2 proteins, which recognise muropeptides released during cell wall turnover, as

well as for destruction of the microorganisms through the hydrolytic activity of lysozyme (Boneca, 2005, Chaput & Boneca, 2007). However, some Gram positive bacteria have developed efficient mechanisms to circumvent these host defences. For instance, *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus pneumoniae* modify their PG by O-acetylation of the C-6 atom of NAM through the action of the OatA protein (Crisostomo *et al.*, 2006, Hebert *et al.*, 2007, Herbert *et al.*, 2007). Some pathogens, such as *Streptococcus pneumoniae and Listeria monocytogenes,* deacetylate the NAG (mostly) and NAM (to a lesser extent) residues into glucosamine or muramic acid through the action of the *pgdA* gene product (Boneca *et al.*, 2007, Vollmer & Tomasz, 2000). These modifications have been shown to result in escaping Nod1 and Nod2 surveillance and/or increased resistance to lysozyme.

The structure of *S. suis* PG has not yet been reported. However, a recent study identified a *S. suis* homolog of the *S. pneumoniae pgdA* gene which was highly upregulated during the interactions of *S. suis* with porcine brain microvascular endothelial cells, suggesting that this bacterium might be able to modify its PG by N-deacetylation (Fittipaldi *et al.*, 2007a). In this study, we determined the *S. suis* PG composition and compared it to that of a Δ*pgdA* isogenic mutant. We demonstrate that *S. suis* N-deacetylates its PG through the action of the *pgdA* gene product and show that the deletion of this gene has a significant impact on the virulence traits of this pathogen.

Results

Fine structure of *S. suis* PG.

The fine structure of *S. suis* PG has never been reported. To determine it, we used the classical method, i.e. isolation and purification of the SDS-insoluble cell wall, removal of teichoic acids by hydrofluoric acid, release of muropeptides by the muramidase cellosyl, separation of muropeptides by high-performance liquid chromatography (HPLC), and matrix assisted laser desorption-ionization-time of flight (MALDI-TOF) mass spectrometry (MS) of the collected muropeptide fractions. Based on previous reports on the amino acids present in the PG of streptococci (Schleifer & Kandler, 1972) the chromatographic behaviour of the muropeptides and their molecular masses, the structures of 13 muropeptides isolated from *S. suis* PG could be proposed with high confidence. The PG of *S. suis* had mainly D-isoglutamine in position 2 and L-

lysine in position 3 of the peptide. The cross-links did not contain an interpeptide bridge frequently found in streptococci. Thus, the muropeptide profile of *S. suis* appeared relatively simple (Fig. 1A), with three major compounds accounting for >75% of the total material, the monomer Tri (the disaccharide tripeptide, compound 1) (Fig. 1B), the dimer TetraTri (bis-disaccharide tetratripeptide, compound 7), and the trimer TetraTetraTri (tris-disaccharide tetratetratripeptide, compound 15). Minor compounds identified had either non-amidated D-glutamate at position 2 (compounds 2 and 8) or a free tetrapeptide or pentapeptide instead of the prevailing tripeptide at the acceptor site of the peptide (compounds 5, 6, 13, 14). Other minor muropeptides had lost a GlcNAc residue (compounds 3, 10, 17), indicating the previous activitiy of a PG glucosaminidase, or a GlcNAcMurNAc moiety (compound 12) due to the activity of a PG amidase. These major and minor muropeptides accounted together for >93% of the total UV-absorbing material (Supplemental Table 1).

Strikingly, the classical method for PG analysis failed to detect any deacetylated muropeptides in the PG of *S. suis* wild-type (WT) field strain 31533, despite the presence of an intact *pgdA* N-deacetylase gene in its genome (see below). Thus, if present at all, deacetylated muropeptides were expected in very small quantities. Therefore, we analysed the muropeptide mixture on a linear ion trap-Fourier transform (LTQ-FT) mass spectrometer which has superior sensitivity and mass accuracy. LTQ-FT-MS detected all 13 major and minor muropeptides from the previous HPLC-MALDI-TOF analysis (Table 1). In addition, 7 new muropeptides were identified including the trimer TetraTetraTetra (compound 19), the tetramer TetraTetraTetraTri (compound 20) and two additional muropeptides with losses of two GlcNAc residues (compounds 11 and 18). Interestingly, LTQ-FT-MS identified the deacetylated versions of all three major muropeptides (compounds 4, 9, 16, Fig. 1B and Table 1). Deacetylated muropeptides were identified in very low quantities, probably lower than 1% of the total muropeptides, preventing determination of the site of deacetylation (NAG or NAM). However, the observed mass intensities of these deacetylated muropeptides were well above the signal noise (Table 1).

The *pgdA* gene product is responsible for N-deacetylation of *S. suis* PG.

N-deacetylation of PG has been shown to occur through the action of the *pgdA* gene product in some Gram-positive species (Boneca et al., 2007, Meyrand *et al.*, 2007, Vollmer &

Tomasz, 2000). A *S. suis pgdA* homolog has recently been identified (Fittipaldi et al., 2007a). Genomic searches at the 4 currently available *S. suis* sequenced genomes available at the NCBI (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/S_suis/), and the DOE Joint Genome Institute (http://genome.jgi-psf.org/draft_microbes/strsu/strsu.home.html) databases, as well as Southern hybridizations using specific probes revealed that *pgdA* is the only putative PG N-deacetylase encoded by *S. suis* (data not shown). The *S. suis pgdA* gene comprises 1398 bp and seems to be transcribed from a putative promoter located 30 bp upstream of the beginning of the coding sequence (identified using the Bprom software at http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) (Fig. 2A). A region of dyad symmetry that might function as a Rho-independent transcription terminator was detected 11 bp downstream the stop codon using the Findterm program (http://www.softberry.ru/berry.phtml?group=programs&subgroup=gfindb&topic=findterm).
The predicted translated sequence of the *S. suis pgdA* gene showed strong homology to previously reported PgdA proteins, including the presence of all the conserved catalytic residues and metal ligand amino acids previously reported for *S. pneumoniae* PgdA (Blair *et al.*, 2005) (Fig. 2B). In order to investigate the involvement of *S. suis pgdA* in PG N-deacetylation we inactivated the gene via a double crossover event, generating a mutant strain in which the *pgdA* gene was replaced by a spectinomycin (Sp) resistance cassette (*aad9* gene). Inactivation of *pgdA* had no major consequences on growth either in normal laboratory medium (Fig. 3A) or in the presence of 10 % porcine or foetal bovine sera (data not shown). In addition, the Δ*pgdA* mutant strain presented a normal morphology and was as encapsulated as the WT strain (Fig 3B). However, when we prepared PG from the Δ*pgdA* mutant and analysed it as described above for the parent strain, no N-deacetylated muropeptides were found (Table 1), showing that N-deacetylation of *S. suis* PG is achieved through the action of the *pgdA* gene product.

S. suis is susceptible to lysozyme _in vitro_ and inactivation of the _pgdA_ gene does not result in an increased susceptibility.

In some Gram positive species N-deacetylation of NAG and/or NAM residues has been shown to confer resistance to lysozyme (Boneca *et al.*, 2007, Meyrand *et al.*, 2007, Vollmer & Tomasz, 2000). Since *S. suis* modifies its PG by N-deacetylation, we sought to evaluate whether this modification results in resistance against this hydrolytic enzyme. We therefore performed a

comparison of the sensitivity to lysozyme of both the *S. suis* WT and Δ*pgdA* mutant strains. Growth of WT *S. suis* was found to be inhibited by lysozyme in a dose-dependent manner. This behaviour was observed both when lysozyme was added at the beginning of the stationary phase of growth (Fig. 4) or at the mid-log phase (data not shown). Growth of *S. suis* was inhibited at concentrations (5 μg/ml) which were much lower than those not affecting growth of several other Gram-positive species such as *S. pneumoniae* (80 μg/ml) and *L. monocytogenes* (10 μg/ml), probably reflecting the low percentage of N-deacetylated muropeptides in its PG. Although recorded values of $OD_{600\ nm}$ were slightly lower than those of the WT strain, the Δ*pgdA* mutant behaved essentially as the WT strain at all the concentrations tested. Since the $OD_{600\ nm}$ assay does not prove lysozyme-mediated killing, we performed plating and colony counts for every condition and time tested. Results showed a strong correlation between the reported decrease in $OD_{600\ nm}$ and loss of viability of *S. suis* (data not shown).

The virulence of the Δ*pgdA* mutant is severely impaired in the murine model of infection.

Components of the *S. suis* cell wall have been proposed to play an important role in the pathogenesis of infection of this pathogen (Higgins & Gottschalk, 2005). In order to evaluate if PG N-deacetylation contributes to the virulence of *S. suis* we performed an experimental infection of CD1 mice using a recently described model of infection (Dominguez-Punaro *et al.*, 2007). Results of the trial showed that most animals in the WT group presented severe clinical signs associated with septicaemia and septic shock, such as depression, swollen eyes, weakness and prostration during the first 72 h post-inoculation (pi). Several mice died from septicaemia in this group. From day 5 pi, some animals in the WT group developed clinical signs associated with *S. suis* meningitis in the mouse (Dominguez-Punaro *et al.*, 2007), such as hyperexcitation, episthotonus, opisthotonus, bending of the head and walking in circles. In strong contrast, mice in the Δ*pgdA* group did not present any major clinical sign associated with *S. suis* infection during the trial, with the exception of slight depression following inoculation which subsided 48 h pi (24 h pi in some cases). No mouse died in this group and significant differences in the mortality rate were noted between the WT and Δ*pgdA* groups (LogRank test, $P < 0.05$) (Fig. 5A). *S. suis* was isolated at high titres for several days (> 1 x 10^7 CFU/ml in some cases) from blood samples (Fig. 5B) and, at euthanasia, from organs such as the liver and spleen of septicaemic animals in the WT group (data not shown). In addition, isolation from the brain and cerebrospinal fluid of animals showing clinical signs of meningitis demonstrated the presence of

S. suis at high titres in both samples (data not shown). In strong contrast, titres in blood following inoculation were lower on average in the Δ*pgdA* mutant group and decreased rapidly. After day 3 pi *S. suis* could was not isolated from this tissue, even if an enrichment of the sample was performed, suggesting that the bacterium had been cleared from circulation (Fig. 5B). In addition, at euthanasia, the Δ*pgdA* mutant could not be isolated from the liver, spleen or brain of any animal in the mutant group (data not shown). No macroscopic lesions associated with *S. suis* were observed in this group at post-mortem examination.

Induction of key cytokines involved in septic shock is abolished in mice inoculated with the Δ*pgdA* mutant.

Differences in PG composition between the WT and Δ*pgdA* mutant strains might result in a dissimilar ability to induce production of several cytokines, as demonstrated for *L. monocytogenes* (Boneca *et al.*, 2007). On the other hand, it has been suggested that an exacerbated inflammatory response leading to septic shock is responsible for death at early stages of the infection of most mice infected with *S. suis* (Dominguez-Punaro *et al.*, 2007). Since we did not observe clinical signs associated with septic shock in mice inoculated with the Δ*pgdA* mutant, we studied kinetically the ability of this strain to induce cytokine production in the mouse. Analysis of the response showed that production of both interferon-γ (IFN-γ) and interleukin (IL)-6 was severely impaired in animals which received the Δ*pgdA* mutant in comparison to those inoculated with the WT strain. In fact, mice that received the WT strain presented an important peak of both IFN-γ and IL-6 at 6 h pi followed by a gradual return to basal levels at 48 h pi. In contrast, animals in the mutant group did not show production of these cytokines above basal levels (Fig. 6A and 6B). Animals in the WT group also showed augmented production of IL-1β up to 48 h pi, whereas the Δ*pgdA* mutant induced very low amounts of this cytokine (Fig. 6C). On the other hand, production of tumour necrosis factor-α (TNF-α) was essentially similar in mice inoculated either with the WT or Δ*pgdA* mutant strains (data not shown). We also studied the chemokine response. Induction of both KC RANTES chemokines was abolished in the Δ*pgdA* mutant group while in the the WT group animals reached peak production between 6 and 12 h pi to return to baseline at 48 pi (Fig. 6D and 6E). Levels of monocyte chemotactic protein-1 (MCP-1) were similar in both groups during the 12 first hours pi, but, thereafter, this chemokine abruptly dropped in the mutant group while it persisted in the WT group up to 36 h pi (Fig. 6F). Overall, with the exception of TNF-α, the Δ*pgdA* mutant

failed to stimulate the production of pro-inflammatory cytokines and chemokines at all the time points tested. On the other hand, the observed production of these inflammatory mediators in the WT group was in perfect agreement with a previous report (Dominguez-Punaro *et al.*, 2007).

The Δ*pgdA* mutant is killed by porcine neutrophils.

It has been shown that in the absence of a specific humoral response, well encapsulated *S. suis* can avoid killing by phagocytes and persist in blood (Chabot-Roy *et al.*, 2006, Charland *et al.*, 1998, Smith *et al.*, 1999). However, our *in vivo* results suggested that the impaired virulence of the Δ*pgdA* mutant may result from its reduced ability to survive in this tissue. Based on previous results in *L. monocytogenes* that showed diminished ability of a Δ*pgdA* mutant to resist killing by phagocytic cells (Boneca et al., 2007), and taking into account that *S. suis* rapidly disappeared from blood, we postulated that clearance of the organism was primarily driven by neutrophils. Indeed, neutrophils are significantly increased in pigs infected with *S. suis* virulent strains and are usually predominant in lesions caused by this pathogen (Salles *et al.*, 2002, Sanford, 1987). When we compared *in vitro* killing of the WT and Δ*pgdA* mutant strains by porcine neutrophils, under our conditions, in the absence of opsonising antibodies but in the presence of normal complete porcine sera, the WT strain resisted the bactericidal effect of porcine neutrophils. On the other hand, suggesting a potential contribution of PG N-deacetylation to this resistance, the Δ*pgdA* mutant strain was efficiently eliminated at rates similar to those observed for an unencapsulated mutant. At the end of the assay, more than 20% of the Δ*pgdA* mutant bacteria were killed by neutrophils compared to less than 5% of the WT strain (Fig. 7).

The Δ*pgdA* mutant is attenuated in the porcine model of infection.

To confirm the observed impaired virulence of the Δ*pgdA* mutant, we conducted a trial in the pig, which is the natural host of *S. suis*. To this end, we used a well-standardised infection model that utilises the aggressive intravenous route of inoculation but provides repeatability (Berthelot-Herault *et al.*, 2001). Animals in the sham-inoculated group did not present any clinical signs during the entire extent of the trial. On the other hand, severe clinical signs such as depression, prostration, lameness and shaking were observed in all 10 animals inoculated with the WT strain. Four pigs died or were sacrificed for ethical reasons within 24 h pi and 4 others at

day 2 pi (Fig. 8). One of these pigs developed clinical signs of meningitis, including convulsions, episthotonus, opisthotonus and bending of the head. At euthanasia, macroscopic lesions typical of *S. suis* infection were found in most animals infected with the WT strain. Fibrin deposits were observed at the pleura and the pericardium of most animals in this group. In addition, all pigs inoculated with the WT group presented at least one swollen articulation with fibrin deposits and excess of synovial liquid. Additionally, some animals in the WT showed damage at the meninges consistent with meningitis. In comparison, animals inoculated with the Δ*pgdA* mutant strain showed less severe clinical signs during the trial. No animal presented shaking or convulsions in this group and no cases of meningitis were recorded. However, depression, prostration and lameness were common during the first 24 h pi. Mortality was also observed in this group, as 4 pigs were sacrificed for strict ethical reasons within the first 3 days pi (Fig. 8). Differences between groups regarding mortality were nonetheless significant (LogRank test, $p<0.05$). The remaining animals in the mutant group recovered starting from day 3 pi (day 2 pi in some cases) and survived until the end of the trial. Examination at necropsy did not reveal major damages to tissues or organs in this group, with the exception of swollen articulations in some animals. Hyperthermia was observed in all pigs infected with either the WT or Δ*pgdA* strains at 24 h pi and no major differences were observed between groups. Temperatures returned to normal values in surviving animals after day 3 in the Δ*pgdA* group and the 2 surviving inoculated animals in the WT group. *S. suis* could be isolated from the blood of all surviving pigs in both groups 48 h pi. However, pigs infected with the WT strain showed higher bacterial counts (1×10^9 CFU/ml in some cases) than those infected with the Δ*pgdA* mutant (average of 5×10^6 CFU/ml). Bacterial isolation from different organs revealed a high bacterial colonization of the liver, the spleen and (in some cases) the brain of pigs in the WT group. In contrast, isolation from the brain was negative in the Δ*pgdA* group, although the bacterium was found in the liver and spleen of most animals in this group. Similar to the blood, bacterial load in organs was lower in pigs inoculated with the Δ*pgdA* mutant compared with animals inoculated with the WT strain.

The *pgdA* gene is upregulated upon interaction with porcine neutrophils and within the host.

In vitro results obtained in this study showing a high sensitivity of the WT strain to lysozyme seem incompatible with the observed outcomes of the experimental infections of both mice and swine as well as with the reported ability of *S. suis* to initiate and establish infections in other hosts (Gottschalk *et al.*, 2007). However, absence of lysozyme resistance *in vitro* might be

explained by a rather inactive *pgdA* gene and a subsequent low extent of PG deacetylation when *S. suis* grows in Todd-Hewitt broth (THB). We therefore hypothesised that *S. suis* might express the *pgdA* weakly in THB and upregulate its expression upon interaction with the host or host cells in order to resist the deleterious effects of lysozyme. To assess this hypothesis, we compared the relative expression levels of the *S. suis pgdA* gene under standard laboratory conditions to that observed upon interaction (as described above) with porcine neutrophils. After reverse transcription, the quantitative PCR (Q-PCR) assays showed that while the expression of the *pgdA* gene by *S. suis* remained essentially unchanged in the mock-infection sample (without neutrophils), it was highly upregulated (mean fold change of 6.7) after 90 min incubation with neutrophils (Fig. 9). We also performed the same analysis from *in vivo* samples (liver and spleen) collected after experimental infection of CD1 mice. Data obtained showed that *S. suis* consistently upregulated the expression of the *pgdA* gene in all the mice tested, with fold changes ranging from 3 to 13, depending on the animal and organ tested (Fig. 9).

Discussion

Even though it has been proposed as an important factor for *S. suis* virulence (Higgins & Gottschalk, 2005), knowledge on the cell wall composition of this pathogen is very limited. A partial structure of *S. suis* (lipo)teichoic acids (LTA) has been reported (Elliott *et al.*, 1977), and very recently it has been shown that *S. suis* modifies LTA by D-alanylation (Fittipaldi *et al.*, 2008). However, PG composition of this bacterium has largely remained elusive. In this study we provide for the first time evidence regarding the composition of this essential polymer in *S. suis,* which was found to be, in several aspects, typical of species within the low GC-content Gram-positive phylum *Lactobacillales*. Like in other streptococci, in *S. suis* mostly D-Gln is found at position 2 of the stem peptide, and an L-Lys residue is present at position 3, which can participate in 4-3 cross-links with neighbouring peptides (Schleifer and Kandler, 1972). However, unlike other streptococci, the PG of *S. suis* lacks an interpeptide bridge but contains direct L-Lys-D-Ala cross-links. Consistently, database searches revealed the absence in *S. suis* of homologues of genes encoding for known peptide branching enzymes of the Fem transferase or ATP-grasp families (Bellais *et al.*, 2006, Filipe & Tomasz, 2000) (data not shown). About 67% of the peptides are present in dimeric or trimeric cross-links (Supplemental Table 1). *S. suis* PG has a low proportion of pentapeptides and tetrapeptides indicating high activities of DD- and LD-carboxypeptidases in this species. Some of the minor muropeptides correspond to modifications

of canonical structures, suggesting the presence of glucosaminidase, amidase and N-deacetylase activities. While the enzymes responsible for the two former activities remain to be identified, N-deacetylation could be linked to the *S. suis pgdA* gene product. To fully ascribe this link, we tried to restore the WT phenotype by complementation of the Δ*pgdA* mutant strain with several different constructions and *E. coli-S. suis* shuttle vectors but, perhaps because it has deleterious effects in *E. coli,* we failed to obtain recombinant plasmids carrying the *S. suis pgdA* gene (data not shown). However, repeated generation of Δ*pgdA* mutants (3 additional independent mutants, Δ*pgdA* 2, 3 and 4, Supplemental Table 3) and consistent verification of a key phenotype such as neutrophil killing for each of them (Supplemental Figure 1) suggest that the occurrence of genetic events elsewhere in the genome other than inactivation of *pgdA* during the procedures for mutant isolation are unlikely. In addition, the genetic organization of the region (Fig. 2A) suggests that polar effects are highly improbable.

S. suis is an important swine pathogen and a zoonotic agent causing septicaemia and meningitis in both pigs and humans (Gottschalk et al., 2007). In order to establish an infection *S. suis* must first overcome the innate immune response of the host. One important mediator of this response is lysozyme. However, some bacterial species resist lysozyme activity by means of PG N-deacetylation and it has been proposed that the level of this modification may correlate with the level of resistance (Hebert et al., 2007, Vollmer *et al.*, 2008b). Consistently with this suggestion, the *S. suis* WT strain, which N-deacetylates PG during growth in normal laboratory culture medium to a very little extent, was sensitive to lysozyme at concentrations much lower than those allowing growth of *S. pneumoniae* and *L. monocytogenes* (Boneca et al., 2007, Vollmer & Tomasz, 2000). Furthermore, we did not observe any major differences between *S. suis* WT and Δ*pgdA* mutant strains regarding resistance to the action of this enzyme *in vitro*. In fact, these results may be explained by an already low degree (probably < 1 % of total muropeptide) of PG N-deacetylation of the WT strain. By comparison, resistance to lysozyme in *S. pneumoniae* correlated with over 80% of N-deacetylated glucosamine residues (Vollmer & Tomasz, 2000). In *L. monocytogenes* the extent of N-deacetylation was lower, but still very important, with up to 50 % of N-deacetylated muropeptides (Boneca *et al.*, 2007). In this regard, the demonstration that *S. suis* upregulates the expression of the *pgdA* gene upon interaction with neutrophils as well as during growth *in vivo* constitutes an interesting and unprecedented finding of this work. Since it is apparent that the *S. suis pgdA* gene product is responsible for PG

N-deacetylation, it is tempting to speculate that this increased expression of the *pgdA* gene correlates with a higher degree of PG N-deacetylation under these conditions that, in turn, results in enhanced resistance to lysozyme produced by neutrophils. However, in view of the fact that it is technically impossible to prepare PG from these samples in amounts and purity suitable to perform HPLC and LTQ-FT mass spectrometry, we are unable to provide conclusive evidence for this hypothesis.

Results of the experimental infections carried out in this study using the Δ*pgdA* mutant suggest that PG N-deacetylation is a major factor in the virulence of *S. suis*. Overall, the infection outcome in the mouse clearly indicates that the Δ*pgdA* mutant is severely hampered in its ability to inflict damage to the host. The most important consequences of PG N-deacetylation are increased survival of *S. suis* at early stages of the infectious process and an increased ability of the pathogen to survive in blood. Interestingly, there was a complete absence of septic shock signs in mice inoculated with the Δ*pgdA* mutant and the production of inflammatory mediators was completely abrogated in mice inoculated with the Δ*pgdA* mutant, while, in perfect agreement with a previous study (Dominguez-Punaro *et al.*, 2007), an exacerbated T helper-1 inflammatory response in mice inoculated with the WT strain was observed. Different results were obtained in a previous study with *L. monocytogenes* showing that the absence of PG N-deacetylation does not result in muropeptides unable to activate the NF-κB pathway, but, instead, in enhanced activation of that pathway in a Nod1- and Nod2-dependent manner (Boneca *et al.*, 2007). With the results obtained in our study we thus propose that, rather than an impaired ability of the unmodified PG to induce exaggerated inflammation, the observed basal levels of pro-inflammatory cytokines in mice inoculated with the Δ*pgdA* mutant might result from the rapid clearance of the mutant by neutrophils (low levels of bacteraemia) along with an irrelevant activation of macrophages, lymphocytes and NK cells in this process.

In spite of the fact that the Δ*pgdA* mutant was able to induce clinical signs in some pigs, conclusions obtained for the mouse may be extended to the pig. Indeed, mortality observed in the Δ*pgdA* mutant group may be explained, at least in part, by the fact that we have prioritised the use of the highly aggressive intravenous route of administration and a high dose of inocula in order to use a validated model of infection (Berthelot-Herault *et al.*, 2001). Nevertheless, in contrast to the WT group, pigs that survived septic shock in the Δ*pgdA* mutant group noticeably recovered and it was apparent that the Δ*pgdA* mutant was less fit to survive in blood. This

inability to persist in blood might also be important for later stages of the infection. Firstly, colonisation of organs was hampered in the mutant group in both the mouse and pig. In addition, it has been shown that if *S. suis* fails to induce acute fatal septicaemia but the level of bacteraemia remains high, CNS disease may appear afterwards (Dominguez-Punaro *et al.*, 2007, Segura *et al.*, 2006). Interestingly, and in agreement with its observed rapid clearance from blood, we did not record any case of meningitis in neither model with the Δ*pgdA* mutant. On the other hand, meningitis was responsible for the death of 35 % of mice and 10 % of pigs in the respective WT groups.

Enhanced clearance of the Δ*pgdA* mutant strain from blood might be attributed to an increased susceptibility of the mutant to killing by neutrophils, as shown in this study. Interestingly, neither the WT or Δ*pgdA* mutant strains showed differences in resistance to the action of cationic antimicrobial peptides such as polymixin B, magainin II, colistin, human neutrophil peptide (HNP)-1 and HNP-2 (Supplemental Table 2), functional homologues of which are secreted by porcine neutrophils. We speculate that differences observed in killing by neutrophils between strains might primarily be explained by the enhanced resistance of the WT strain to lysozyme secreted by neutrophils, due to increased PG N-deacetylation after induction of the *pgdA* gene. On the other hand, since our killing assay does not differentiate between intra and extracellular killing it is difficult to estimate if increased killing of the Δ*pgdA* mutant correlate with increased phagocytosis. However, since the Δ*pgdA* mutant is as encapsulated as the WT strain and, on the other hand, it has been shown that capsular material interferes with the uptake of *S. suis* by phagocytes (Chabot-Roy *et al.*, 2006, Charland *et al.*, 1998, Segura *et al.*, 2004, Smith *et al.*, 1999), enhanced phagocytosis of the mutant might be unlikely. Interestingly, it has been suggested, although not proved, that an encapsulated *S. suis* Δ*dltA* mutant might be killed by porcine neutrophils extracellularly in the so called neutrophil extracellular traps (NETs) (Fittipaldi *et al.*, 2008). Since neutrophils, in addition to delivering their complex antibiotic arsenal into the phagosome, also discharge their lysozyme-rich specific granules extracellularly in the NETs (Cho *et al.*, 2005, Mollinedo *et al.*, 2006) it might be postulated that the Δ*pgdA* mutant may also be killed through the action of this enzyme after being trapped in these extracellular structures. Further experiments are needed to confirm this hypothesis.

In conclusion, we have determined the muropeptide composition of *S. suis* and showed that this bacterium, through the action of the *pgdA* gene product, modifies its PG by means of

N-deacetylation. We showed that this PG modification, which is probably enhanced *in vivo*, greatly contributes to the virulence of *S. suis* in both the murine and porcine models of infection. The results of this study strongly suggest that PG N-deacetylation is a major virulence factor of this swine pathogen and zoonotic agent.

Experimental procedures

Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids used in this study are listed in Supplemental Table 3. *S. suis* strains were grown in THB or in Todd-Hewitt agar (THA) (Becton Dickinson, Sparks, MD, USA) at 37°C under 5% CO2. *Escherichia coli* strains were cultured in Luria-Bertani broth or agar medium (Becton Dickinson) at 37°C for 18 h. When necessary, antibiotics (purchased from Sigma-Aldrich, Oakville, On, Canada) were added to culture media at the following concentrations: for *E. coli*, kanamycin, 50 µg/ml; chloramphenicol (Cm), 10 µg/ml and Sp, 50 µg/ml; for *S. suis*, Cm, 5 µg/ml and Sp, 100 µg/ml.

Purification of PG.

S. suis cell wall was prepared from cultures in the exponential growth phase as described for *S. pneumoniae* (Garcia-Bustos & Tomasz, 1987, Severin *et al.*, 1997) Wall teichoic acid and acid-labile modifications (such as O-acetylation) were removed by treatment with hydrofluoric acid to obtain PG (Severin *et al.*, 1997).

Preparation and HPLC-analysis of muropeptides.

PG (ca. 1.25 mg/ml) was stirred with 25 µg/ml cellosyl (kindly provided by Hoechst, Frankfurt, Germany) in 20 mM sodium phosphate pH 4.8 for 24 h at 37°C. Then, a second 25 µg/ml-aliquot of cellosyl was added and the incubation continued for another 24 h. The sample was boiled for 10 min and centrifuged at room temperature for 15 min at 16000 × g. The supernatant was recovered and reduced with sodium borohydride as described (Glauner, 1988). The reduced muropeptides were separated on a 250×4.6 mm, 3 µm Prontosil 120-3-C18 AQ reversed-phase column (Bischoff, Leonberg, Germany) as described (Glauner, 1988), with the exception of the running buffers. Buffer A was 10 mM sodium phosphate pH 6.0 supplemented

with 13 µl/l of 10% sodium azide; buffer B was 10 mM sodium phosphate pH 6.0 with 30% methanol. The eluted muropeptides were detected at 205 nm.

MALDI-TOF analysis of muropeptides.

Muropeptides eluted from the C18 column were collected, concentrated in a SpeedVac to 10-20 µl, and acidified with 0.1% trifluor acetic acid (TFA). The sample was then zip-tipped (C-18 material, Millipore, UK) according to the manufacturer's standard protocol, eluted from the zip-tip with 3 µl of 50% acetonitrile, 0.1% TFA and mixed with α-cyano-4-hydroxycinnamic acid matrix prior to spotting on a target plate. MALDI-TOF analysis was done on a Voyager-DE STR mass spectrometer (Applied Biosystems, Warrington, UK) operating in positive ion reflectron mode over the mass range (m/z) of 500 to 3000.

Preparation of muropeptides and LTQ-FT-MS analysis.

Different conditions for muropeptide preparation were used for samples subjected to LTQ-FT-MS analysis. PG (ca. 0.8 mg/ml) was digested with 13 µg/ml cellosyl in 20 mM ammonium acetate buffer, pH 4.8, for 16 h at 37°C. The sample was boiled for 10 min and centrifuged at room temperature for 15 min at 16.000×g. The sample (1 µl) was applied on a C18-reversed phase self-packed 100×0.5 mm column and eluted with a linear gradient of acetonitrile in water, 0.1% formic acid. The eluted fractions were sprayed directly into an LTQ-FT mass spectrometer (Thermo, Bremen, Germany) operating in the positive ion mode. The details of the method will be published elsewhere (Bui *et al.*, manuscript in preparation).

DNA manipulations.

Restriction enzymes, DNA-modifying enzymes and DNA polymerase were purchased from GE Healthcare (Piscataway, NJ, USA) or Takara Bio (Otsu, Shiga, Japan) and used accordingly to the manufacturer's recommendations. Minipreparations of recombinant plasmids from, and transformation of *E. coli* were performed by standard procedures (Sambrook *et al.*, 1989). Transformation of *S. suis* was carried out as previously described (Takamatsu *et al.*, 2001a). *S. suis* genomic DNA was isolated by the guanidium thiocyanate method (Pitcher *et al.*, 1989). Southern hybridizations were performed by the procedures described previously (Sekizaki *et al.*, 2001). For preparation of probes, DNA fragments were labelled with digoxigenin

(DIG) using the DIG-PCR labeling mixture (Roche Diagnostics, Laval, QC, Canada) according to the manufacturer's instructions. Oligonucleotide primers (Invitrogen, Burlington, ON, Canada) are listed in Supplemental Table 4.

Construction of the knockout vector for gene replacement.

DNA fragments were amplified from genomic DNA of *S. suis* strain 31533 by PCR using the primer sets 4197F-PG plus 4985R-PG (left arm) or 6276F-PG plus 6838R-PG (right arm) (Supplemental Table 4). A Sp resistance cassette (*aad9* gene) was amplified from pSmall with primers specF3 and specR. All three primer sets introduce unique restriction sites. PCR amplicons were digested using the appropriate restriction enzymes, and sequentially ligated in the order left arm-Sp cassette-right arm using T4 DNA ligase. The resulting fragment was amplified by PCR using primers 4197F-PG and 6838R-PG, cloned into pCR4 using the TOPO TA PCR cloning kit (Invitrogen) following the manufacturer's instructions and propagated in *E. coli*. The insert was then cut out with HindIII and BamHI and recloned into the HindIII and BamHI sites of the thermosensitive vector pSET-5s, which carries the Cm resistance gene *cat* (Takamatsu *et al.*, 2001b), generating knockout vector p5ΔpgdA.

Generation of *S. suis* Δ*pgdA*.

Procedures for selection of mutants by allelic exchange via double crossover were described previously (Takamatsu *et al.*, 2001b). Briefly, *S. suis* strain 31533 was transformed with p5ΔpgdA, and the cells were grown at 28°C in the presence of Cm and Sp selection. Bacteria at the mid-logarithmic growth phase were diluted with THB containing Sp and grown at 28°C to early logarithmic phase. The cultures were then shifted to 37°C and incubated for 4 h. Subsequently, the cells were spread on THA containing Sp and incubated at 28°C. Temperature-resistant Sp-resistant colonies were screened for loss of vector-mediated Cm resistance to detect putative mutants which had exchanged their WT allele for a genetic segment containing the *aad9* gene as a consequence of homologous recombination via a double crossover. Finally, the genetic organization of the resulting Δ*pgdA* mutant was verified by PCR and by Southern hybridisation (data not shown). Three additional independent Δ*pgdA* mutants (named Δ*pgdA* 2, 3, and 4 respectively, Supplemental Table 3) were generated using the same strategy.

Transmission electron microscopy.

ON cultures of *S. suis* WT strain 31533 or mutant strain Δ*pgdA* were mixed with specific polyclonal serum anti-*S. suis* serotype 2 and incubated at room temperature for 1 h, followed by fixation in cacodylate buffer (0.1M cacodylate, 5%, v/v, glutaraldehyde 0.15%, w/v, ruthenium red, pH 7.2) for 2 h. Cells were then immobilised in 4% (w/v) agar and post-fixed ON at 4°C in 2% (v/v) osmium tetraoxyde. Samples were dehydrated in graded series of ethanol and embedded in Spurr low-viscosity resin. Thin sections were post-stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Model 420, Philips Electronics, The Netherlands).

Lysozyme and cationic antimicrobial peptide sensitivity.

S. suis WT and Δ*pgdA* mutant were grown in THB to which was added hen egg lysozyme (final concentrations ranging from 2 to 50 µg/ml; Sigma-Aldrich). Growth was monitored by following the optical density at 600 nm and by plating serials dilutions onto THA followed by CFU enumeration after ON incubation. Assays for sensitivity to cationic antimicrobial peptides were carried out in sterile 96-well microtiter plates. Logarithmic-phase *S. suis* cells were adjusted to approximately 10^4 CFU/ml in 100 µl THB containing one of the following antimicrobial compounds (purchased from Sigma-Aldrich) in serial dilutions: Colistin (0 to 200 µg/ml), polymyxin B (0 to 300 µg/ml), magainin II (0 to 45 µg/ml), HNP-1 (0 to 15 µg/ml) and HNP-2 (0 to 15 µg/ml). Plates were incubated for 24 h at 37°C. The MIC was defined as the lowest antimicrobial concentration yielding no detectable bacterial growth by $OD_{600\,nm}$ measurement.

Killing by porcine neutrophils.

Blood samples were collected by venous puncture from high health status pigs which tested negative by ELISA (Lapointe *et al.*, 2002) for *S. suis* serotype 2. Cell populations were separated by Ficoll-Hypaque (GE Healthcare) density gradient centrifugation and neutrophils isolated by sedimentation in 6% dextran, as decribed (Chabot-Roy et al., 2006). Contaminating erythrocytes were removed by lysis with 0.83% ammonium chloride. Neutrophils were resuspended in RPMI 1640 medium (Invitrogen) supplemented with 10% of heat-inactivated porcine serum at a final concentration of 5×10^6 cells/ml. Bacteria (WT or Δ*pgdA* mutant strains at approx. 1×10^4 CFU/ml) were opsonised with complete normal porcine serum for 30 min at 37°C and then mixed in microtubes with neutrophils at 5×10^6 cells/ml. The mixture was

incubated for 90 min at 37°C under 5% CO_2. Under these conditions bacteria are not toxic to neutrophils (Chabot-Roy *et al.*, 2006). After incubation cells were lysed with sterile water and viable bacterial counts on THA were performed.

Experimental infection of mice.

All experiments involving animals were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care. For virulence studies a total of 30 female CD1 mice aged 6 weeks old (Charles River laboratories, Wilmington, MA, USA) were used. At day 0, animals were divided in two groups. Group 1 was inoculated by intraperitoneal injection of 1 ml of a *S. suis* strain 31533 suspension at 5 x 10^7 CFU/ml, while group 2 received the same dose of the mutant strain Δ*pgdA* using the same route of administration. Mice were monitored 3 times a day during 10 days for clinical signs and assigned clinical scores as previously described (Dominguez-Punaro *et al.*, 2007). Surviving animals in both groups were sacrificed at day 10 pi. Blood was collected daily from the tail vein and at euthanasia by cardiac puncture and used to evaluate bacterial load in this tissue by plating onto sheep blood agar plates and enumeration after ON incubation. Bacterial colonisation of the liver, the spleen and the brain of infected animals was also evaluated. Briefly, small pieces of these organs weighing 0.5 g were trimmed, placed in 500 µl of phosphate buffered saline (PBS), pH 7.3, and homogenised. Thereafter, 50 µl of the suspensions were plated as described above. In addition, an enrichment of the samples was carried out by inoculation of 300 µl of homogenised organ samples or 100 µl of blood into THB, followed by ON incubation at 37°C and subsequent dilution and plating onto sheep blood agar plates as described above.

Analysis of the systemic immune response in mice.

A total of 42 female CD1 mice aged 6weeks old (Charles River) were used to study the systemic immune response. The WT strain (1 ml of a 5 x 10^7 CFU/ml suspension) was inoculated as described above to 18 mice. The same number of mice received the Δ*pgdA* mutant (1 ml of a 5 x 10^7 CFU/ml suspension). Finally, 6 mice were sham-inoculated. At defined intervals (3h, 6h, 12 h, 24 h, 36 h and 48 h) pi infected mice were sacrificed (3 animals per time for those inoculated with the WT or mutant strains, and 1 mouse per time for those sham-inoculated), and blood was collected by cardiac puncture. The bacterial dose and time points used were

those reported in a previous study (Dominguez-Punaro *et al.*, 2007). For validation of the conditions, we previously performed an additional independent study with a limited number of animals and time points which showed results consistent with those obtained in the major experiments described in the manuscript (data not shown). Plasma was recovered from blood samples by centrifugation and the levels of IFN-γ, IL-6, IL-1β, TNF-α, MCP-1, KC and RANTES were determined using a liquid multiarray system (Luminex Molecular Diagnostics, Toronto, ON, Canada). Commercial Multiplex coated beads and biotinylated antibodies, as well as the Beadlyte microtiter 96 well filter plates were obtained from Millipore, (Billerica, MA, USA). Each multiplex assay was performed in duplicate following the manufacturer's specifications. Data were collected using the Luminex-100 system Version IS 2.2 and analysed by MasterPlex Quantitation Software (MiraiBio, San Francisco, CA, USA). Standard curves for immune response mediators were obtained using the standards supplied by the manufacturer.

Analysis of the *pgdA* gene expression.

Total RNA was prepared from 1 ml of an early stationary phase *S. suis* culture in THB ("untreated control", approx 10^7 CFU/ml) using RNAwiz (Ambion, Austin, TX, USA) according to the manufacturer's instructions. To analyze expression of *pgdA* upon interaction of *S. suis* with neutrophils, the WT type strain was treated as described above in the killing section and allowed to interact with porcine neutrophils for 90 min. Five tubes of the mixture of neutrophils and bacteria were then pooled and total RNA was prepared as described above. In addition, 5 tubes of a "killing mock-infection" (RPMI plus 10 % porcine serum without neutrophils, as described in the killing section) were pooled and total RNA prepared from using RNAwiz. For analysis of *in vivo* expression, 5 female CD1 mice aged 6weeks old were inoculated as described above with 1 ml of a *S. suis* strain 31533 suspension at 5 x 10^7 CFU/ml. Animals were sacrificed 24 h pi. Pieces of spleen and liver (weighing approx 0.2 g) were collected and disrupted with 0.1 mm glass beads using a mini bead beater (Biospec Products, Bartlesville, OK, USA) and total RNA prepared with RNAwiz, as described. All RNA samples were treated with TurboDNAse (Ambion), and absence of contaminating DNA was verified by PCR using primers AROA-F and BA9 (Supplemental Table 4), which target the *aroA* gene. RNAs were quantified by measurement of absorbance at 260 nm and their integrity verified by visualization on 1% denaturing agarose gels. Synthesis of cDNAs was performed in triplicate using SuperScript II (Invitrogen) with random hexamers (Roche, Laval, Qc, Canada). Q-PCR was carried out by using the QuantiTect

SybrGreen PCR Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. For each sample, a no-reverse transcription reaction was run as a control. For each Q-PCR run, to normalize for the amount of sample cDNA added to each reaction, the Ct value of the *pgdA* gene was subtracted by the *Ct* value of the endogenous control *rpoD* gene (delta *Ct* = *Ct pgdA* –*Ct rpoD*), and then, for a comparison between the "untreated control" (bacteria grown in THB) group and the "treated" groups (upon interaction with neutrophils, "killing mock infection", *in vivo* samples), the delta *Ct* values of the treated groups were subtracted by the delta *Ct* value of the untreated control (delta delta *Ct* = delta *Ct* treated group – delta *Ct* untreated control). The fold changes were calculated by the formula of $2^{-\text{delta delta Ct}}$ (Livak & Schmittgen, 2001). Primers were SSU1448-FQ and SSU1448-RQ for *pgdA* and RPDO-F and RPDO-R for *rpoD* (Supplemental Table 4).

Experimental infection of pigs.

A total of 24 high-health-status pigs (ages 4 to 5 weeks) which tested negative by ELISA (Lapointe et al., 2002) for *S. suis* serotype 2 were used. Strict biosecurity measures were implemented to avoid undesirable contamination of the pigs; these included an air filtration system and airlocks for each unit. Pigs were divided in 3 groups. Animals in groups 1 (N=10) and 2 (N=10) were inoculated by intravenous injection of 1 ml of 1 x 10^8 CFU/ml of *S. suis* WT strain 31533 or mutant Δ*pgdA*, respectively. Group 3 (N=4) were sham-inoculated animals. Clinical signs and presence of *S. suis* in blood were monitored during the trial. Surviving animals in all 3 groups were sacrificed 5 days pi and examined for pathological lesions. Bacteriological isolation from different organs (liver, spleen, lungs, heart and articulations) was performed essentially as described above for the mice.

Statistics.

Unless otherwise specified, all the data were expressed as means ± standard deviations. Unless otherwise specified, data were analysed by two-tailed, unpaired *t* test and all assays were repeated at least three times. For data analysis of the systemic immune response and bacteremia in mice the Mann-Whitney test was used. For *in vivo* virulence experiments, survival was analysed with the LogRank test. For all tests, a value of $P < 0.05$ was considered as the threshold for significance.

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Article III Tables

Table VII. Article III, Table 1. Muropeptides detected by LTQ-FT mass spectrometry.

Muropeptide			WT		*ΔpgdA*	
No	proposed structure	theoretical neutral mass (Da)	neutral mass (Da)	signal intensity (A.U.)[1]	neutral mass (Da)	signal intensity (A.U.)[1]
1	Tri	823.3811	823.3793	157,566	823.3795	124,676
2	Tri (Glu)	824.3889	824.3644	n. q.[2]	824.3646	n. q.
3	Tri (-G)	620.3017	620.3012	5,961	620.3012	4,212
4	Tri (deAc)	781.3705	781.3708	450	n.d.[3]	n.d.
5	Tetra	894.4182	894.4171	17,168	894.4173	15,822
6	Penta	965.4553	965.4541	9,763	965.4549	4,835
7	TetraTri	1699.7887	1699.7874	108,961	1699.7871	59,454
8	TetraTri (Glu)	1700.7966	1700.7580	n.q.	1700.7598	n.q.
9	TetraTri (deAc)	1657.7781	1657.7766	5,488	n.d.	n.d.
10	TetraTri (-G)	1496.7093	1496.7084	5,551	1496.7083	3,095
11	TetraTri (-2G)	1293.6299	1293.6287	10,318	1293.6291	3,716
12	TetraTri (-GM)	1221.6088	1221.6080	3,021	1221.6086	903
13	TetraTetra	1770.8259	1770.8249	2,588	1770.8261	1,288
14	TetraPenta	1841.8630	1841.8620	3,834	1841.8626	2,013
15	TetraTetraTri	2576.1964	2576.1952	17,734	2576.1995	7,391
16	TetraTetraTri (deAc)	2534.1858	2534.1944	139	n.d.	n.d.
17	TetraTetraTri (-G)	2373.1170	2373.1174	1,813	2373.1226	544
18	TetraTetraTri (-2G)	2170.0376	2170.0369	2,191	2170.0407	780
19	TetraTetraTetra	2647.2335	2647.2275	84	2647.2398	19
20	TetraTetraTetraTri	3452.6040	3452.6084	1.972	3452.6113	708

[1] the noise of the signal is ≈10 A.U.

[2] not quantified because the signal partly overlaps with the isotope signal of the amidated compound

[3] n.d. not detected

Table VIII. Article III, Supplemental Table 1. Quantification and MALDI-TOF-MS analysis of the muropeptides separated by HPLC.

Muropeptide		Area %		Positive mass (Da) from collected peaks[2] reduced, Na^+ form	
No	proposed structure[1]	WT	Δ*pgdA*	measured	calculated
1	Tri	18.8	20.9	848.40	848.39
2	Tri (Glu)	2.2	2.4	849.38	849.40
3	Tri (-G)	n.q.[3]	n.q.	623.33[4]	623.33[4]
4	Tri (deAc)	n.d.[5]	n.d.	n.d.	806.38
5	Tetra	2.6	2.7	919.46	919.42
6	Penta	1.5	1.7	990.51	990.46
7	TetraTri	46.0	46.7	1726.86	1726.81
8	TetraTri (Glu)	2.5	1.9	1727.88	1727.82
9	TetraTri (deAc)	n.d.	n.d.	n.d.	1684.80
10	TetraTri (-G)	3.3	4.1	1523.77	1523.73
11	TetraTri (-2G)	n.d.	n.d.	n.d.	1320.65
12	TetraTri (-GM)	1.1	0.3	1246.67	1246.61
13	TetraTetra	1.7	1.3	1797.97	1797.85
14	TetraPenta	1.9	2.0	1869.02	1868.88
15	TetraTetraTri	11.7	10.2	2605.34	2605.23
16	TetraTetraTri (deAc)	n.d.	n.d.	n.d.	2563.22
17	TetraTetraTri (-G)	n.q.[6]	n.q.[6]	2402.27	2402.15
18	TetraTetraTri (-2G)	n.d.	n.d.	n.d.	2199.07
19	TetraTetraTetra	n.d.	n.d.	n.d.	2676.27
20	TetraTetraTetraTri	n.d.	n.d.	n.d.	3483.65

[1] see Fig. 1B

[2] peaks were collected from the WT sample

[3] not quantified, co-elutes with Tri

[4] mass of the H^+ form

[5] n.d., not detected

[6] **n.q., not quantified, co-elutes with TetraTetraTri**

Table IX. Article III, Supplemental Table 2. Sensitivity of the *S. suis* WT and Δ*pgdA* mutant strains to the action of selected antimicrobial peptides.

		MICs (μg/ml)	
Peptide (origin)	Net charge	WT	Δ*pgdA*
Colistin (*Bacillus colistinus)*	+5	50	50
Polymyxin B (*Bacillus polymyxa*)	+5	75	75
Magainin II (claw frog skin)	+4	45	45
HNP-1 (human)	+3	6	6
HNP-2 (human)	+3	5	5

Table X. Article III, Supplemental Table 3. Bacterial strains and plasmids used in this study.

Bacterial strains	Relevant characteristics	Source
E. coli Top ten	General strain for cloning. F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
S. suis 31533	Serotype 2 field strain. Highly virulent	(Vanier *et al.*, 2004)
S. suis Δ*pgdA*	Derived from strain 31533. Deletion of the *pgdA* gene	This work
S. suis Δ*pgdA* 2	Derived from strain 31533. Deletion of the *pgdA* gene	This work
S. suis Δ*pgdA* 3	Derived from strain 31533. Deletion of the *pgdA* gene	This work
S. suis Δ*pgdA* 4	Derived from strain 31533. Deletion of the *pgdA* gene	This work
S. suis BD102	Derived from strain 31533. Deletion of the promoter of the *aro* operon. Unencapsulated.	(Fittipaldi et al., 2007)
Plasmids		
pCR4	*E. coli* vector for cloning of PCR fragments	Invitrogen
pSmall	*E. coli-S. suis* shuttle vector. SpecR. Source of *aad9*	P. Willson (unpublished)
pSET5s	Thermosensitive suicide vector for *S. suis* mutagenesis. CmR (*cat*)	(Takamatsu et al., 2001)
p5ΔpgdA	pSET5s carrying the construction for allelic exchange	This work

Table XI. Article III, Supplemental Table 4. Oligonucleotide primers used in this study. Restriction sites are in bold.

Primer name	Sequence (5' – 3')	Restriction site
4197F-PG	GACGGG**TGGTCAA**GCTTTTGAATA	HindIII
4985R-PG	CCATTATGCCGAGCAAGG**AGATCT**TG	BglII
6276F-PG	GTAACAGACTTACT**CTGCAG**TCCTC	PstI
6838R-PG	GTGAACGTTT**GGATCC**TCTACGTAA	BamHI
SPECF2	TTATCAGGAT**AGATCT**TCGTTCGTGA	BglII
SPECR	AAAGTGTTTC**CTGCAG**TTTTTCAA	PstI
COMPPGDA-F3	GGATTTAAG**GGTACC**CGTCGCAAGGA	KpnI
COMPPGDA-R	ACCAG**GGATCC**TACTAACATGATTC	BamHI
AROA-F	AACGTGACCTACCTCCGTTG	
AROA-R	CGGTCATCGTAGAATTCGAGT	
SSU1448-FQ	TTCTCTCTGTACTTGCTCCC	
SSU1448-RQ	GGTCGCTCTAACCTTTGATG	
RPOD-F	TCTTTCAAATACATGCGGACTG	
RPOD-R	ATTCCATTTACGCTTGATGCTG	

Article III Figures

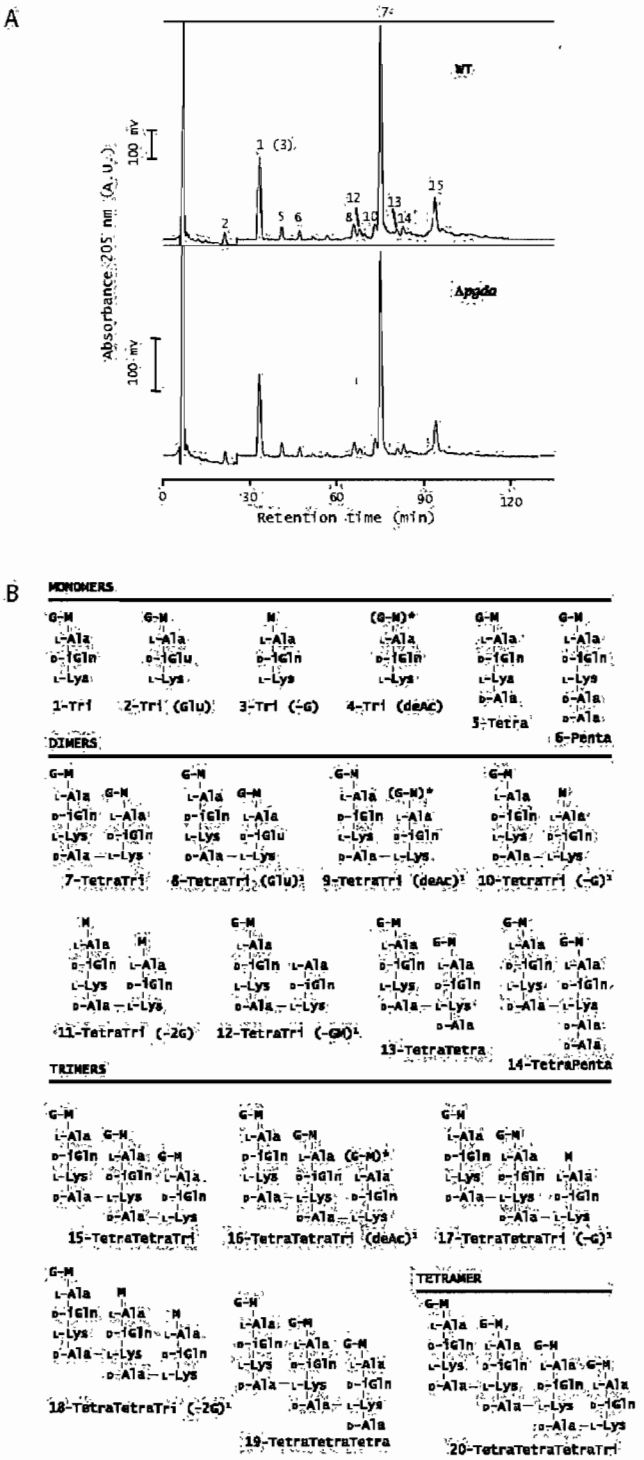

Figure 21. Article III, Figure 1. *S. suis* muropeptide composition and proposed structures.
A. Muropeptide composition of *S. suis* WT and Δ*pgdA* mutant. The numbers correspond to the structures shown in B. The profiles of both strains showed similar muropeptide composition.

However, see Table 1 and the text for differences between the WT and Δ*pgdA* mutant. B. Proposed structures for *S. suis* muropeptides. G indicates *N*-acetylglucosamine residue; M indicates *N*-acetylmuramic acid residue. Asterisks indicate deacetylation of G or M. In compounds marked with [1] it is not known which of the residues is modified.

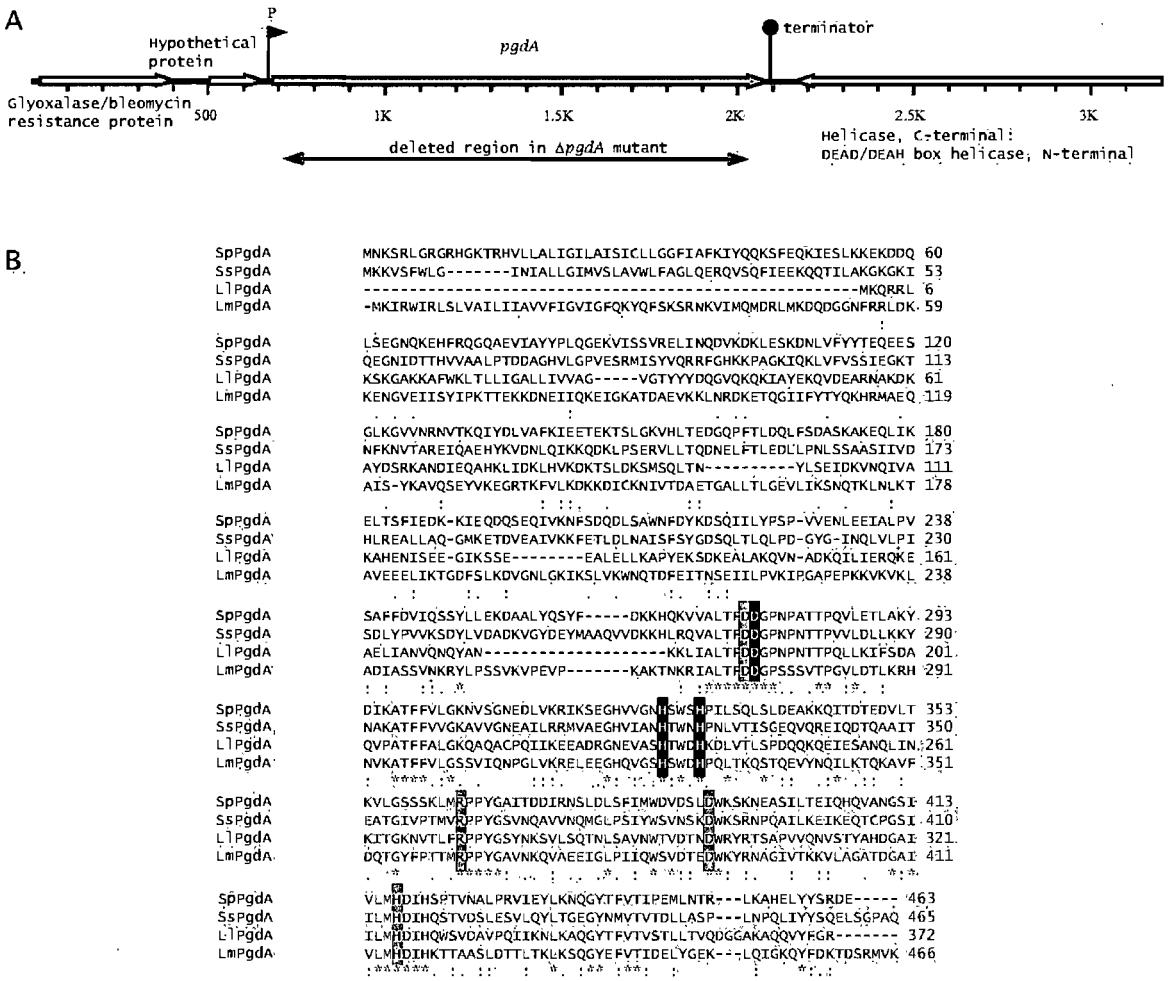

Figure 22. Article III, Figure 2. Genetic organization of the *pgdA* region and Clustal alignment of the predicted *S. suis* PgdA.

A. Genetic organization of the *S. suis pgdA gene* showing the predicted putative strong promoter (indicated by P) and the Rho-independent putative terminator. The region deleted in the Δ*pgdA* mutant (replaced by gene *aad9*) is also indicated. See the text for details B. Sequence alignment of *S. suis* (SsPgdA) and the described *N*-acetylglucosamine deacetylases from *S. pneumoniae* (SpPgdA) *L. lactis* (LlPgdA) and *L. monocytogenes* (LmPgdA). Sequences were aligned using the CLUSTALW software (http://www.ebi.ac.uk/Tools/clustalw/). Residues in grey and black boxes indicate the conserved catalytic residues and metal ligand amino acids characterised for *S. pneumoniae* PgdA (Blair et al., 2005), respectively. Asterisks below alignment indicate identical aminoacids, colons strongly conserved aminoacids and dots weakly conserved aminoacids.

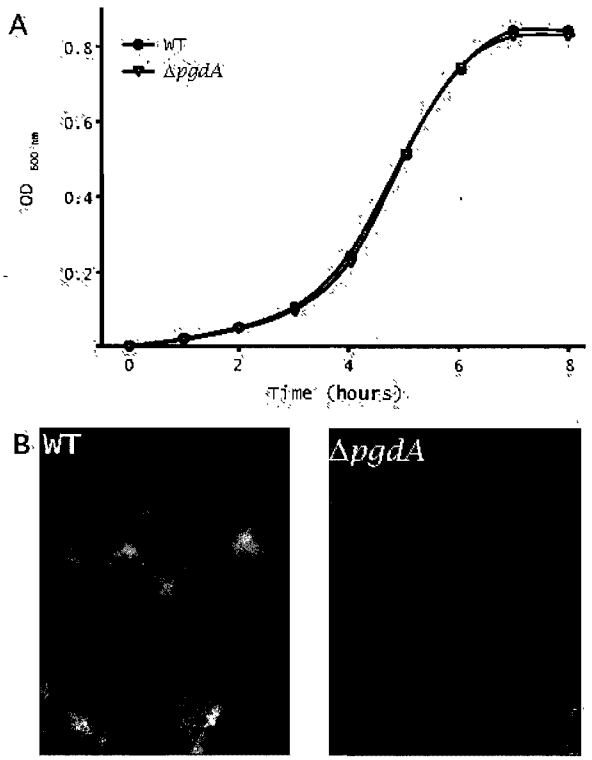

Figure 23. Article III, Figure 3. Growth curves and morphology of the WT and Δ*pgdA* mutant.
A. Growth curves of *S. suis* WT and Δ*pgda* mutant strains. The Δ*pgdA* mutant (open triangles) grew similarly as the WT parent strain (closed circles) under normal laboratory conditions. B. Morphology of the WT (left) and the Δ*pgdA* mutant (right) strains. Transmission electron microscopy showed that both strains were well separated and surrounded by a thick polysaccharide capsule. Original magnification : 30 000X.

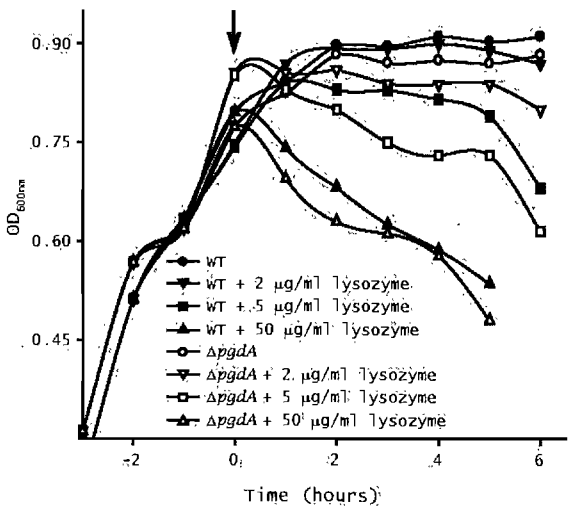

Figure 24. Article III, Figure 4. Lysozyme sensitivity of *S. suis* WT and Δ*pgdA* mutant strains.
The WT and the Δ*pgdA* mutant strains were grown at 37 C, until the beginning of stationary phase of growth was reached, at which point, indicated by the arrow, lysozyme was added at different concentrations. Growth was monitored by measuring absorbance at 600_{nm}. Experiments were performed at least 3 times. Error bars are not shown for simplicity.

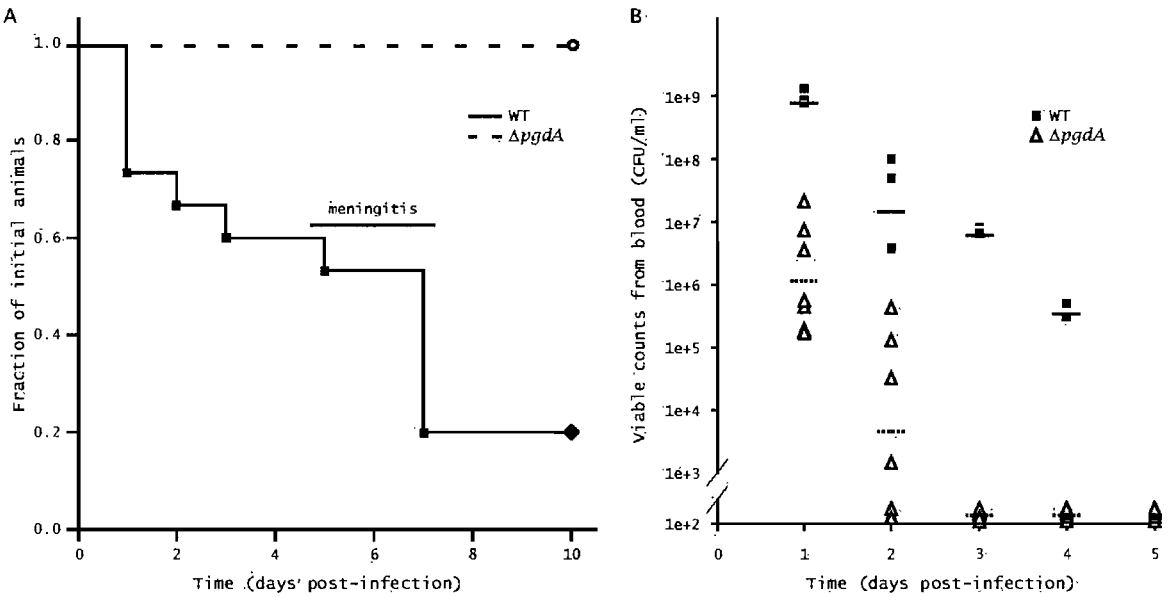

Figure 25. Article III, Figure 5. Results of the WT and Δ*pgdA* mutant infection in mice.
A. Survival of mice inoculated with the WT (solid line) or the Δ*pgdA* mutant (dotted line) strains. All the mice in the Δ*pgdA* mutant (N=15) group survived, while 80% of the WT group (N=15) died from septicaemia or meningitis. Significant difference in survival were noted (LogRank test, $P<0.05$). B. Kinetics of bacterial clearance from blood in the WT and Δ*pgdA* mutant. The Δ*pgdA* mutant was isolated at lower titers than the WT strain following inoculation and was undetectable in this tissue from day 3 pi. Squares and triangles represent values from individual mice. The horizontal lines indicate the median for each group (WT: solid line; Δ*pgdA* mutant: dashed line). Significant differences in isolation from blood were noted between groups from day 1 to day 4 pi (Mann-Whitney, $P<0.05$).

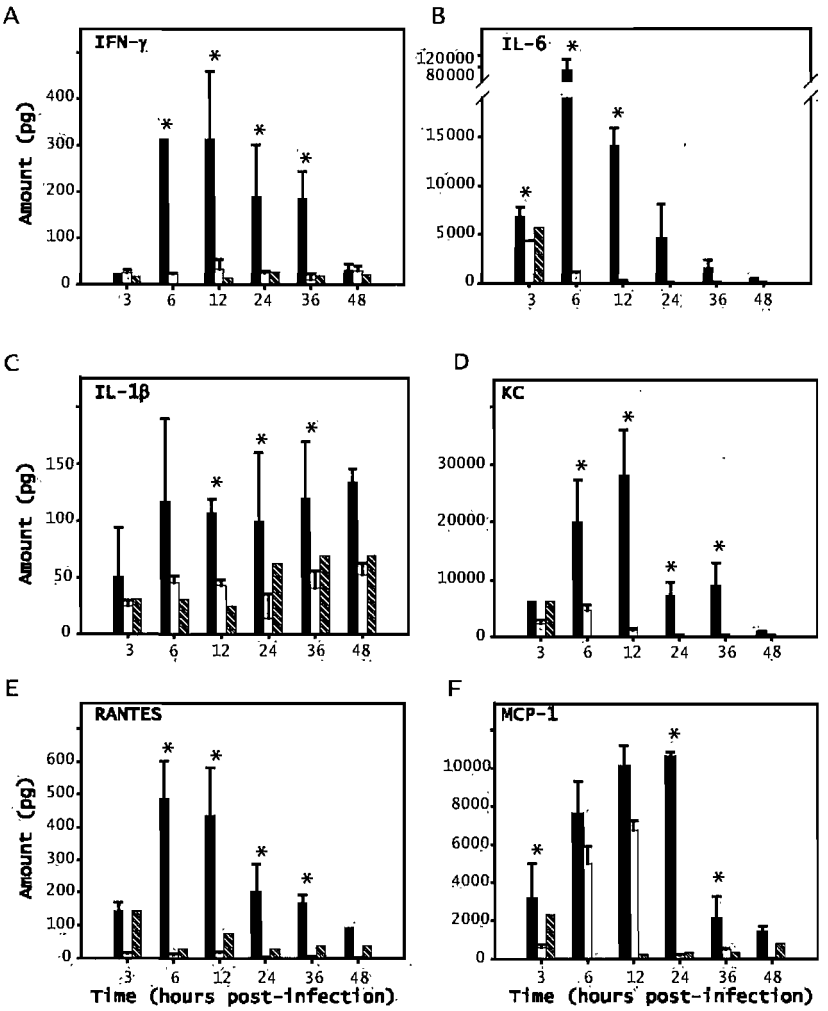

Figure 26. Article III, Figure 6. Production of inflammatory mediators by mice inoculated with the WT strain (black bars, 3 animals per time), the Δ*pgdA* mutant (white bars, 3 animals per time) or sham-inoculated mice (dashed bars, 1 animal per time).

A. IFN-γ; B. IL-6; C. IL-1β; D. KC; E. RANTES; F. MCP-1. Significant differences (Mann-Whitney test $P<0.05$) were observed between the WT and the Δ*pgdA* mutant for all the inflammatory mediators at different time points (indicated by asterisks). Error bars indicate the standard errors of the median. See the text for further details.

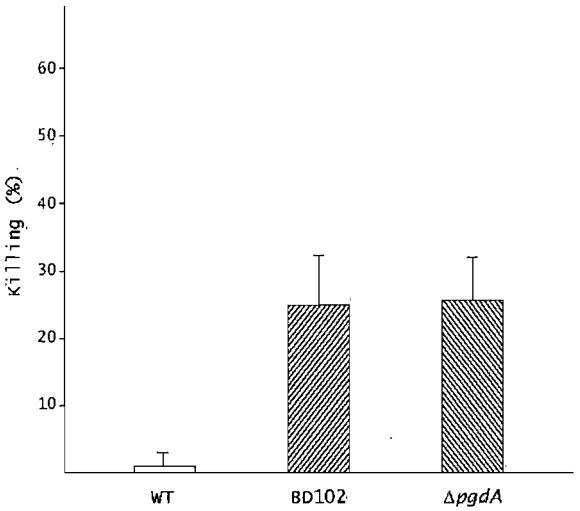

Figure 27. Article III, Figure 7, Percentage of bacteria killed after 90 min incubation with porcine neutrophils.
The level of killing of the Δ*pgdA* mutant was similar to that observed for an unencapsulated mutant BD102 and significantly higher (t-test, P<0.05) than that of the WT strain. The different strains were opsonised with complete porcine sera before incubation. The experiment was repeated at least 5 times. Error bars indicate standard deviations calculated from biological repetitions.

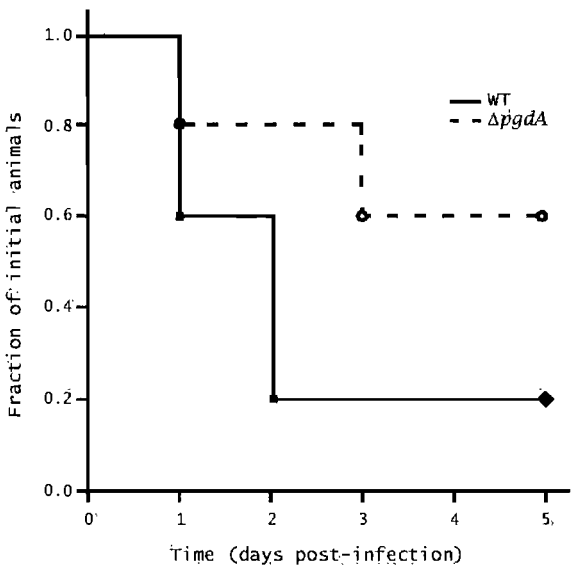

Figure 28. Article III, Figure 8. Results of the WT and Δ*pgdA* mutant infection in swine.
Survival of pigs inoculated with the WT (solid line, N=10) or the Δ*pgdA* mutant (dotted line, N=10). Sham inoculated animals are not shown for simplicity. Significant differences in survival were noted, LogRank test, P<0.05. The survival rate of pigs in the Δ*pgdA* mutant group was 60%. In the WT group 80 % of the animals did not survive the trial. See the text for further details.

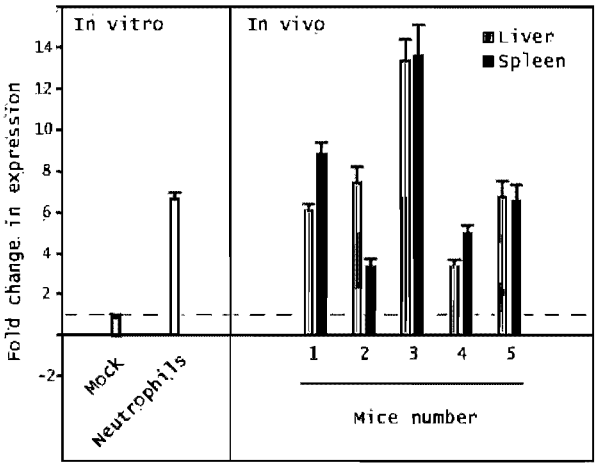

Figure 29. Article III, Figure 9. Q-PCR analysis of the level of expression of the *S. suis pgdA* gene upon 90 min interaction of the bacteria with porcine neutrophils, or *in vivo* in the liver and spleen of experimentally infected mice.

The listed fold changes are relative to expression of *pgdA* when the bacteria were grown in THB (defined as 1 and shown by the dashed line). Fold changes were calculated using the delta-delta *Ct* method (Livak & Schmittgen, 2001). Please see the materials and methods section for further details. For *in vitro* samples (neutrophils), bars show the mean ± standard deviation of at least three biological repetitions. For *in vivo* samples, bars represent individual animals (mean ± standard deviation of three technical repetitions).

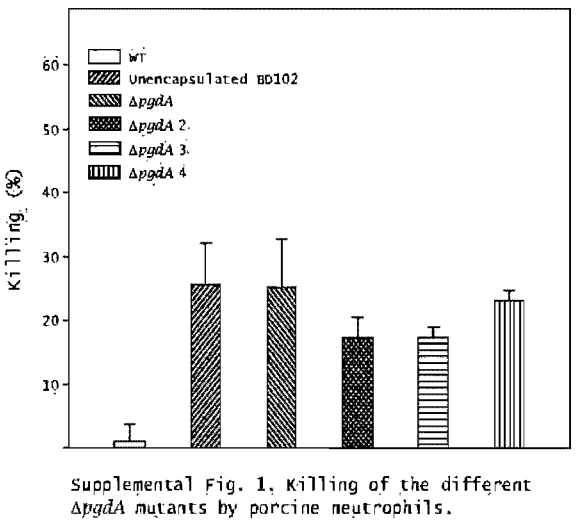

Figure 30. Article III, Figure S1. Killing of the different Δ*pgdA* mutants by porcine neutrophils.

IV. DISCUSSION

Current knowledge on *S. suis* virulence factors.

As with many other extracellular bacteria, *S. suis* produces a polysaccharide capsule that allows for resistance to complement-mediated lysis and phagocytosis by leukocytes. At the beginning of this research project, the single most critical virulence factor described for *S. suis* was the CPS (Chabot-Roy et al., 2006; Charland et al., 1998; Smith et al., 1999a). Still today, the CPS is the best characterized *S. suis* virulence factor (Gottschalk et al., 2007). However, most *S. suis* avirulent strains are encapsulated, and many avirulent well encapsulated strains are rapidly cleared from the circulation (Gottschalk and Segura, 2000). In addition to the CPS, a fibronectin/fibrinogen-binding protein (de Greeff et al., 2002b) seems to be partially involved in virulence. Other factors are not essential for virulence, are absent from some virulent strains, or have not yet been properly studied due to the unavailability of knockout mutants (Gottschalk et al., 2007). Recently, an OFS has been described and shown to be critical for the virulence of *S. suis* (Baums et al., 2006). However, like the CPS, this factor is present in both virulent and avirulent *S. suis* strains (Takamatsu et al., 2008b). Some other proposed putative virulence factors have been shown to be dispensable for the virulence of *S. suis*. This is the case for MRP and EF proteins (Smith et al., 1993; Smith et al., 1992), as well as for suilysin, (Gottschalk et al., 1995; Jacobs et al., 1994). Indeed, MRP and EF do not seem to confer virulence attributes directly, as shown by the use of isogenic mutants (Smith et al., 1996). On the other hand, suilysin has been identified as a toxic factor for various cell types (Charland et al., 2000; Segura and Gottschalk, 2002; Vanier et al., 2004; Vanier et al., 2008b). This toxin also has the ability to interfere with complement-mediated opsonophagocytosis (Chabot-Roy et al., 2006). Nevertheless, isogenic mutants lacking suilysin were shown to be as virulent to pigs as the respective parent strain (Allen et al., 2001; Lun et al., 2003). Though, there is a positive association between the presence of these proteins and virulence in Eurasian strains of serotype 2. Furthermore, avirulent strains expressing MRP, EF and suilysin have not been described so far. On the other hand, the absence of one or more of these proteins is not necessarily associated with a lack of virulence. Indeed, besides experimental trials with isogenic mutants, some European and most Canadian virulent isolates do not produce these factors (Berthelot-Herault et al., 2000; Gottschalk et al., 1998; Quessy et al., 1995; Segers et al., 1998). Recent data (Article V, Appendix) suggest that the situation in the United States is similar to that in Canada regarding the lack of production of these virulence markers for serotype 2 strains.

Whether a single isolate of *S. suis* should be classed as virulent or avirulent is still a matter of controversy (Berthelot-Herault et al., 2005; Gottschalk and Segura, 2000; Gottschalk et al., 2007). Despite increasing research in the last four years that led to the genome sequencing of several *S. suis* strains, as well as the recent identification of novel putative virulence determinants (see the Review of the literature), the paragraph above still represents, to a certain extent, the current knowledge on *S. suis* virulence. Therefore, the need for the discovery of novel *S. suis* virulence factors was beyond doubt justified. The development of the *in vitro* model of *S. suis*-porcine BMEC interactions (Vanier et al., 2004) offers an excellent chance to interrogate *S. suis* gene expression during a key step in meningitis development such is BBB crossing. Indeed, BMEC are one of the major cellular types forming the BBB, the impermeable barrier that only certain bacteria, including *E. coli*, *N. meningitidis*, *M. tuberculosis*, GBS, *S. pneumoniae*, *L. monocytogenes* and *S. suis* are able to cross (Kim, 2006). Therefore, genes expressed by *S. suis* during its interactions with porcine BMEC may potentially be considered important virulence factors of this swine pathogen and zoonotic agent.

To that end, in this research project we used an efficient molecular approach (SCOTS), which selects for and identifies differences in bacterial gene expression and the vitro model of *S. suis* adhesion/invasion of porcine BMEC. In a considerable step forward for our understanding of the pathogenesis of the *S. suis* infection, in this research project we identified twenty-eight genes that were upregulated by *S. suis* during its interactions with porcine BMEC. Selected genes were further studied and the role they play in *S. suis* virulence was evaluated.

Validity of the SCOTS approach and general considerations about the identified genes

The SCOTS approach has been used with success in several bacterial species (Baltes et al., 2007; Baltes and Gerlach, 2004; Bauer et al., 2008; Daigle et al., 2001; Dozois et al., 2003; Faucher et al., 2005; Faucher et al., 2006; Graham and Clark-Curtiss, 1999; Graham et al., 2002; Hou et al., 2002; Jin et al., 2008; Liu et al., 2002; Zhu et al., 2008). To the best of our knowledge, Article I, presented in the body of this thesis, is the first report of the use of the SCOTS approach for analysis of differential gene expression in streptococci. Results presented in Article I clearly demonstrate that SCOTS is also suitable for the elucidation of gene expression in this important bacterial genus. To fully realize the importance of the use of the SCOTS approach, it has to be taken into account that at the beginning of this research project, very few molecular tools had

been developed for *S. suis* and most of today's finished/ongoing whole genome sequencing projects had not even been started. Therefore, SCOTS, which is a technique that does not require specialized mutagenesis systems or a detailed knowledge of genome sequence data, provided us with an extraordinary opportunity for seeking novel virulence factors of this pathogen. Indeed, before the present research project, only one genomic approach had been used for the identification of *S. suis* virulence factors (Smith et al., 2001a). In that work, a promoter-trap system (an adapted IVET approach) identified several *S. suis* promoters that were expressed upon iron-starvation conditions or *in vivo* (porcine infection model) (Smith et al., 2001a). Interestingly, in that study, all the promoters identified as being active *in vivo* by the use of the promoter-trap approach were also expressed *in vitro* under standard laboratory growth conditions, a fact that the authors of that study explained by the absence of promoter sequences exclusively expressed *in vivo* (Smith et al., 2001a). However, since a plasmid-based instead of an integrative promoter trap system was used, the results obtained might as well be explained by the inability of that system to detect *in vitro* silent genes due to gene dose effects.

In this research project, by using SCOTS we clearly showed condition-specific differences in *S. suis* gene expression that were confirmed in selected cases by the use of Q-RT-PCR. A comparison between the genes identified by SCOTS and those detected by the use of the promoter-trap approach revealed that there were no overlapping genes between the two *S. suis* studies. This was an interesting finding, because IVET and SCOTS have identified the same genes in some cases (Rediers et al., 2005). However, absence of overlapping genes was not surprising, since only a small number of genes were identified in either study, and, more importantly, the experimental conditions used were essentially different. Therefore, in this research project, the use of the SCOTS approach resulted in original data leading to the identification of several interesting virulence factor candidates, further study of which might provide new insights on the molecular mechanisms that this pathogen might use to cross the BBB. However, it is important to note that based on comparison against the current reports available in the literature, none of the identified genes might be, *per se*, responsible for BBB crossing. As discussed, blood-borne bacteria able to invade the CNS may cross the BBB or the blood-CSF barriers following direct interaction with the luminal side of the cerebral endothelial tissue or choroid plexus epithelial cells. Only a limited number of bacterial pathogens are able to traverse these barriers, a fact that suggests that these organisms have developed specific strategies and possess specific

attributes. However, no specific gene implicated directly in the crossing of BBB, giving clues for a better understanding of this phenomenon, has ever been found by the use of global approaches such as IVET or STM (Kim, 2006). The SCOTS study presented in Article I was no exception, although many of the genes identified may play important roles in virulence. Indeed, the currently acknowledged hypothesis is that crossing of the BBB and/or the blood-CSF barrier is the result of the concerted and synergistic action of many genes. Abolition of the crossing would probably need inactivation of several of them (Autret and Charbit, 2005; Kim, 2006).

As mentioned above, the identification of the twenty-eight genes preferentially expressed upon the interactions of *S. suis* with porcine BMEC may result in a better understanding of how this pathogen causes meningitis. Indeed, several of these genes show a great potential as virulence factor candidates. On the other hand, many genes putatively involved in metabolism were also identified by SCOTS. These genes are often disregarded or at least considered of less interest because they are linked to housekeeping functions and do not encode "genuine" colonization or virulence factors. However, they can provide new information about the metabolism of the microbe that may, in the case of animal pathogens such as *S. suis*, prove useful for vaccine development. Metabolic genes are isolated frequently in practically all IVET, STM and SCOTS studies (Autret and Charbit, 2005; Daigle et al., 2002; Rediers et al., 2005) and their increased expression clearly means that fulfilling nutritional requirements is important. It can nevertheless be considered intriguing that, using our *in vitro* model where nutritional elements are unlimited, a relatively high proportion of SCOTS-identified genes are putatively involved in metabolism. However, it is known that transcriptional activation of most bacterial promoters in their natural environments is not a simple on/off decision, as the expression of cognate genes is integrated in layers of iterative regulatory networks that ensure the performance not only of the whole cell, but also of the bacterial population, in a changing environment (Cases and de Lorenzo, 2005). One environmental signal (whether it is nutritional or physicochemical) can upregulate or downregulate the activity of many promoters, and this regulatory network is known as a regulon. From a mechanistic point of view, regulons involve regulatory proteins that allow different promoters to respond to the same signal. Moreover, a common stimulus can drive entire regulons to act in concert (a stimulon) (Cases and de Lorenzo, 2005). Although we are far from having identified the specific signal(s) that trigger upregulation of SCOTS-identified genes, it can be hypothesized that upon contact with porcine BMEC, in

addition to genes required for adhesion/invasion of these cells, *S. suis* also upregulates other genes required for optimal ecological performance.

The hypothesis mentioned above is also supported by the isolation of other, non-metabolic, genes such as *pgdA*, whose role in *S. suis* virulence will be extensively discussed below. Indeed, this gene has been shown to be primarily involved in resistance to lysozyme, an effector of the innate immune response of the host (Boneca et al., 2007; Vollmer and Tomasz, 2000). Interestingly, the *pgdA* gene was found to be upregulated in the presence of porcine BMEC, even if no lysozyme was present in our *in vitro* model of infection. However, assuming a yet unknown signal(s) and the concept of regulon- or even stimulon-driven expression of *S. suis* genes upon interaction with porcine BMEC, induction of the *pgdA* gene may represent a logical response to an *in vivo* situation where the immune response of the host would be much more relevant than it is *in vitro.* Knowledge on the signal(s) that elicit or repress gene expression in *S. suis* remains so far truly vague, not only for the novel SCOTS-identified genes, but also for almost all of the proposed virulence factors of this pathogen. Even the regulation of the CPS, so far the most characterized critical virulence factor of *S. suis*, remains unknown, despite the identification of the *cps* locus (Smith et al., 1999a; Smith et al., 2000) and previous reports accounting for increased production of capsular material *in vivo* (Quessy et al., 1994). Indeed, data presented in the Appendix of this thesis (Article 4) seem to indicate that regulation of capsule production may perhaps be linked to the aromatic amino acid pathway. Without a doubt, the naive notion that "everything is connected to everything" in biological systems applies also to *S. suis* and emphasizez that further efforts are needed to shed light into the complexity of virulence regulation in this important pathogen.

Putative collagenase

As stated above, some genes identified by SCOTS might be considered potential virulence factor candidates for *S. suis*. Interestingly, at the time of publication of Article I, a report was published describing the use of STM for *S. suis*. In that report, twenty-two transposon mutants were shown to be attenuated in either the pig or mouse model of infection (Wilson et al., 2007). A variety of genes were thus identified, including genes previously found to be essential to the virulence of other organisms (Wilson et al., 2007). These findings have been described in the Review of the literature section of this thesis. As for the promoter trap

approach discussed above, no overlap was found between the SCOTS and STM studies, with the exception of one common gene. Interestingly, the STM study (Wilson et al., 2007) reported the identification of a gene putatively involved in collagen degradation (SSU0457) that had also been isolated using the SCOTS approach in this research project. It has been shown that *S. suis* is able to interact with ECM proteins and to bind, among others, fibronectin, fibrinogen and collagen (Esgleas et al., 2005). On the other hand, *S. suis* has the ability to induce the upregulation of matrix metalloproteinase 9 and metalloproteinase 3 production by human macrophages and porcine CPEC (Jobin et al., 2006; Tenenbaum et al., 2008). One proven action of these enzymes is the digestion of the ECM, an action that may result in tissue destruction and disruption of the BBB and blood CSFbarrier, which may in turn facilitate (further) trafficking of bacteria and leucocytes. However, the direct ability to degrade ECM proteins has not yet been demonstrated for *S. suis*. Therefore, the identification by SCOTS and, in parallel, by STM, of this putative collagenase might be of importance for better understanding the mechanisms used by *S. suis* to reach the CNS. Interestingly, in sequenced strains P 1/7 and 89-1591, the putative collagenase identified by SCOTS is located upstream of a gene putatively encoding a second putative collagenase, in an operon-like organization. It has been suggested that the impairment of BBB barrier function during infection with different *S. suis* strains may depend on proteases produced by this pathogen (Jobin and Grenier, 2003). It is thus tempting to speculate, even if we lack evidence regarding its exact function, that upregulating the expression of this collagenase *in vivo* might be useful for *S. suis* to increase the permeability of the BBB and blood-CSF and therefore contribute to the migration of *S. suis* to the CNS. From the STM study, it seems that the action of this collagenase is essential for full virulence of the pathogen (Wilson et al., 2007). Further assessment of the action of this protein *in vitro* should clarify its involvement in collagen degradation.

Pili

Pili in several Gram positive bacteria have recently been described (Telford et al., 2006). In contrast to those produced by Gram negative bacteria, Gram positive pili are formed by covalent polymerization of pilin subunits in a process that requires a dedicated sortase enzyme (Proft and Baker, 2009). Pilin subunits are added to the fiber by the dedicated Class C sortase, and then are covalently linked to the cell wall peptidoglycan by the housekeeping sortase (Dramsi et al., 2006; Dramsi et al., 2005; Telford et al., 2006). In all Gram positive pili described

so far, the structural pilin subunits, as well as the class C sortases required for polymerization, have been shown to be encoded by genes which are clustered together in discrete loci (Proft and Baker, 2009; Telford et al., 2006). In addition to the dedicated sortase, Gram positive pili clusters comprise at least two genes encoding the pilin subunits, each of which contains an LPXTG (or a variant thereof) amino-acid motif (Proft and Baker, 2009). One of these proteins (known as the major pilin subunit) constitutes the backbone of the pilus, while the second (minor or ancillary pilin subunit) might be located at the tip of the structure and might constitute the adhesin. The exact location of the minor pilin subunit within the pilus structure is yet a matter of discussion (Proft and Baker, 2009). Pili clusters with more than one minor pilin subunit have also been described, and in these cases the exact role of each minor subunit is controversial (Telford et al., 2006). Sometimes, the pili clusters may contain a signal peptidase gene, which, in GAS, has been shown to be required for pili polymerization (Zahner and Scott, 2008).

In *S. suis*, the presence of thin pilus-like structures on the surface of the bacterium has been revealed by electron microscopy (Jacques et al., 1990). In this research project, by the use of the SCOTS approach, we identified for the first time a pili cluster in *S. suis* (Article I). Indeed, SCOTS isolated a gene putatively encoding a signal peptidase homologous to the LepB-type signal peptidases of Gram-negative bacteria. An homologous LepB-type signal peptidase is the first gene in the GBS pilus island 2b (PI-2b), one of the three identified pili islands in this species (Telford et al., 2006). The GBS PI-2b contains five other downstream genes, encoding two LPXTG proteins (putative ancillary pilin subunit and the main pilin subunit), a class C sortase, a third LPXTG protein (ancillary pilin subunit) and a second class C sortase (Telford et al., 2006) [Figure 14, (Article I, Figure 2)]. Interestingly, in *S. suis* sequenced strains P1/7 and 89-1591, two genes encoding LPXTG proteins (highly homologous to the ancillary and main pilin subunits of GBS, respectively) and a gene encoding an undescribed putative class C sortase-like protein were found downstream of the LepB signal peptidase that was identified by SCOTS (Article I, Figure 2). Although the *S. suis* putative pilus island lacks the last 2 genes in comparison to that of GBS, based on the similarity of the genetic organization, the strong homology showed by the LPXTG proteins to the main and ancillary pilin subunits of this latter species and the current proposed mechanism for pili formation in Gram-positive bacteria (Dramsi et al., 2006; Mora et al., 2005), we suggested that a pilus might be formed by the gene products of this island. In addition, since

pili have been very recently shown to be important for GBS adhesion to and invasion of human BMEC (Maisey et al., 2006), we speculated that this putative pilus might participate in *S. suis* adhesion to or invasion of porcine BMEC (Article I). Following the identification of this pilus cluster, a project aimed at further characterizing pili production by *S. suis* has been established. Research is ongoing and some unpublished results showing that the pilus cluster F indeed drives the expression of pili, as well as a short discussion, will be presented in the appendix section of thesis.

D-alanylation of the LTA and the virulence of *S. suis*

LTA is an amphiphilic polymer of poly-Gro-P or polyphosphoribitol, anchored to the cytoplasmic membrane by a glycolipid (Weidenmaier and Peschel, 2008). Each poly-Gro-P or phosphoribitol present on this molecule may be modified by glycosylation or, more often, may contain D-alanine residues (Neuhaus and Baddiley, 2003). One of the SCOTS-identified genes, the gene *dltB* (ssu0597) belongs to an operon comprising four genes, *dltABCD*, which is present in all genomes of low-G+C bacteria determined so far. In all species where this operon has been studied, all four genes are required to catalyze the incorporation of D-alanine residues into the LTA (Neuhaus and Baddiley, 2003). It has been shown that the *dltA* gene encodes the D-alanyl carrier protein ligase DltA which activates D-alanine for ligation to the D-alanyl carrier protein (DltC). The product of the *dltD* gene (DltD) functions in the selection of the carrier protein DltC for ligation with D-alanine (Debabov et al., 2000), while DltB, a membrane protein, has been proposed to be involved in the secretion of D-alanyl-DltC, which would then transfer the D-alanine molecule to the LTA (Figure 11). D-Alanylation of the LTA has important consequences: it allows Gram positive organisms to modulate their surface charge, to regulate ligand binding and to control the electromechanical properties of the cell wall (Archibald et al., 1973; Neuhaus and Baddiley, 2003). In addition, formation of D-alanyl-LTAs has been shown to be required to resist the action of cationic antimicrobial peptides (CAMPs) in *L monocytogenes, S. aureus, S. pneumoniae*, GAS and GBS (Abachin et al., 2002; Kovacs et al., 2006; Kristian et al., 2005; Poyart et al., 2003; Weidenmaier, 2005a). Besides, virulence of mutants deficient in D-alanylation of LTA of GBS, *L. monocytogenes* and *S. aureus* has been shown to be severely impaired in the murine or rabbit models of infection (Abachin et al., 2002; Poyart et al., 2003; Weidenmaier, 2005a).

Before this research project, the D-alanylation of *S. suis* LTAs had not been documented. Indeed, the structure and composition of *S. suis* LTA are so far poorly known. It has been proposed that LTA from *S. suis* may have a backbone structure similar to that of GAS teichoic acid, but with differences in glucosyl substituents: a monosaccharide glucosyl substituent is found in the LTA from *S. suis* instead of the di- or trisaccharide previously postulated as the glucosyl substituent in the teichoic acid of GAS (Elliott et al., 1977). Despite the fact that the structure of the LTA has not yet been revealed, it is known that WT *S. suis* LTA is important for adhesion of this bacterium to porcine BMEC. Indeed, inhibition of the adhesion of *S. suis* to this cellular type was obtained by pre-incubation of porcine BMEC with purified LTA (Vanier et al., 2007).

From our SCOTS results and previous reports in other organisms, we hypothesized that *S. suis* might be able to modulate the degree of D-alanylation of its LTAs by upregulation of the *dlt* operon. We anticipated that this modification may have a profound impact not only for the interactions of this pathogen with porcine BMEC but also at other stages of the infection. Further studies focusing on this operon of *S. suis* were therefore carried out to evaluate this hypothesis. To that end, we inactivated the first gene of the *dlt* operon in a virulent field strain of this pathogen and carried out several *in vitro* and *in vivo* experiments. The results of this research project (Article II) demonstrate that *S. suis* modifies its LTA by D-alanylation and that this modification is dependent on the action of the product of the *dltA* gene and, by extension, of the *dlt* operon. The absence of LTA D-alanylation had no major effect on *S. suis* growth under normal laboratory conditions. On the other hand, previous reports have shown that GBS and GAS Δ*dltA* mutants were either poorly separated or multiseptated in the stationary phase of growth (Kristian et al., 2005; Poyart et al., 2003). However, no major differences were found regarding the morphology of the *S. suis* Δ*dltA* mutant strain when analyzed by transmission electron microscopy. Indeed, the mutant was encapsulated, well separated and presented normal septation.

Results obtained in this research project are consistent with previous reports of inactivation of the *dltA* gene in several streptococcal species (Kovacs et al., 2006; Kristian et al., 2005; Poyart et al., 2003). For instance, addition of D-alanine residues to the LTA greatly improved the resistance of *S. suis* to the action of CAMPs. These compounds, classified into several groups based on their structure and origin, kill bacteria by forming pores in the

cytoplasmic membrane (Taheri-Araghi and Ha, 2007). Changes in the bacterial surface via introduction of positively charged D-alanine residues into the LTA molecules would reduce the global negative charge of the *S. suis* envelope, thus providing the bacterium with a physical mechanism of resistance against the action of CAMPs (Neuhaus and Baddiley, 2003). An early response of the immune system to bacterial infection is the migration of neutrophils to the infected tissue (Nathan, 2006). Neutrophils sense inflammatory mediators, such as IL-8, and translocate from the bloodstream to the site of infection where they initiate mechanisms that help clear infecting bacteria (Nathan, 2006). It has been shown, however, that WT *S. suis* resists killing by porcine neutrophils (unless opsonized by specific antibodies). This resistance has been primarily attributed to the production of a thick polysaccharide capsule that prevents phagocytosis of the bacterium by the polymorphonuclear cells (Chabot-Roy et al., 2006). Consistently, in this research project an unencapsulated mutant strain was effectively eliminated by porcine neutrophils, while the capsulated WT strain resisted killing by these cells even when opsonized with complete porcine serum (Article II). Interestingly, although the Δ*dltA* mutant does not have altered capsule expression, it was also killed by porcine neutrophils under the same experimental conditions.

Opsonization of foreign microbes occurs after complement activation through deposition of C3b and its cleavage fragment iC3b on the microbial surface. Complement receptors on neutrophils engage the bound C3b or iC3b to facilitate phagocytosis (Nizet, 2007). The presence of D-alanyl esters has been shown to diminish purified LTA binding to C1, decreasing activation of the classical complement pathway (Loos et al., 1986). It may be thus hypothesized that the enhanced killing of the Δ*dltA* mutant by porcine neutrophils might be the result of better opsonization resulting in improved phagocytosis. However, in intact bacteria, complement activation by LTA seems to be of minor importance because peptidoglycan itself is a potent activator (Wilkinson et al., 1981). On the other hand, neutrophils are also able to destroy infecting microorganisms in the absence of phagocytosis in the so-called neutrophil extracellular traps (NETs) (Brinkmann, 2004). While this extracellular killing is effective against some Gram positive pathogens (Brinkmann, 2004; Wartha et al., 2007), not surprisingly, other pathogenic bacteria have developed ways to resist killing by neutrophils in NETs (Beiter, 2006; Buchanan, 2006; Wartha, 2007). Interestingly, it has recently been shown in *S. pneumoniae* that absence of LTA D-alanylation results in enhanced extracellular killing in NETs by neutrophils but

not in an increased phagocytosis of this organism by these polymorphonuclear cells (Wartha, 2007). Although our killing assay was not able to discriminate between intra and extracellular killing, taking all these findings together, it may be proposed that the capsulated *S. suis* Δ*dltA* mutant is killed by porcine neutrophils extracellularly, perhaps after being trapped in NETs. In addition, we speculate that this enhanced killing of the *S. suis* Δ*dltA* mutant might be the consequence of the absence of LTA D-alanylation, which results in an increased susceptibility to CAMPs released by neutrophils. Further experiments are needed to evaluate this hypothesis.

The contribution of LTA D-alanylation to virulence varies among different streptococci. For instance, the virulence of a GBS Δ*dltA* mutant was severely impaired in mice (Poyart et al., 2003), while the reduction in virulence of a *S. pneumoniae* Δ*dltA* mutant was less significant (Wartha, 2007), despite the fact that both species showed increased susceptibility to CAMPs and killing by murine or human neutrophils and/or macrophages. These differences may reflect the unique lifestyles and pathogenic features of these bacteria. Although *S. suis* shares certain characteristics with the former two species, its pathogenesis of infection is essentially different (Higgins and Gottschalk, 2006). It is believed that, once in the blood of infected animals, *S. suis* escapes leukocyte killing and, by unknown mechanisms, migrates to different organs where it causes tissue damage which often results in septic shock (Gottschalk et al., 2007; Higgins and Gottschalk, 2006). Results obtained in this research project by evaluating the performance of the Δ*dltA* mutant *in vivo* show that LTA D-alanylation contributes to *S. suis* virulence during the onset of the infection and provides an advantage to the WT strain in the pig, as shown by the higher survival rate of the animals infected with the mutant compared with the WT strain. However, despite this reduction in virulence, bacterial dissemination was not prevented and some pigs developed an exacerbated inflammatory response resulting in septic shock. In addition, we did not observe a clear reduction in the ability of the Δ*dltA* mutant to successfully initiate infection and induce septicemia in mice infected with a high dose of inoculum. Since clearance of the mutant from circulation seems to rely on neutrophil activity, the high dose used to inoculate the animals may explain, at least in part, the results observed. Indeed, it has been proposed that suilysin may affect complement activity and suilysin-producing *S. suis* strains, such as the WT and mutant strains used in this study, have been shown to be toxic to neutrophils at high titers (Chabot-Roy et al., 2006). In addition, since CAMPs activity primarily

occurs at mucosal surfaces, the extremely aggressive intravenous route of administration may have also influenced the clinical onset observed in pigs.

Some of the *in vitro* phenotypes resulting from absence of LTA D-alanylation presented in Article II confirm previous reports in other Gram positive bacteria. Actually, Δ*dltA* mutants in a few Gram positive pathogenic species showed similar or comparable increases in susceptibility to CAMPs and/or killing by neutrophils and/or macrophages as those reported in the present research project for *S. suis*. However, a review of the literature shows that virulence of Δ*dltA* mutants has been tested for some species only. Interestingly, in these cases strong variations in the pathogenic power of these mutants was found between bacterial species, despite the apparent similarity of *in vitro* phenotypes (Abachin et al., 2002; Poyart et al., 2003; Wartha, 2007; Weidenmaier, 2005a). Therefore, drawing conclusions regarding the contribution of LTA D-alanylation to virulence traits of pathogens from previous studies may not be reliable. In this regard, a major contribution to the field of this research project is the *in vivo* data presented. Indeed, we provide truly original data since we have for the first time evaluated the virulence of a Gram positive Δ*dltA* mutant in the context of its natural host by intravenous inoculation of pigs. Given that only a small number of studies have characterized the virulence of Δ*dltA* mutants and, for various valid reasons, these studies have used surrogated models of infection instead of the natural hosts (Abachin et al., 2002; Poyart et al., 2003; Wartha, 2007; Weidenmaier, 2005b), data presented in our work is relevant and contributes to a better comprehension of the contribution of D-alanylation of LTA to the virulence traits of pathogenic Gram positive organisms.

In most scenarios, invasion of the CNS occurs in the context of a systemic disease and typically follows bacterial dissemination via the bloodstream (Kim, 2006). Extracellular bacteria have developed efficient strategies (LTA D-alanylation seems to be an important one) to solve the biological problem of how to escape elimination by host defenses such as phagocytes and serum proteins. As a result, these pathogens typically achieve a high-density bacteremia that is important for CNS invasion (Autret and Charbit, 2005; Kim, 2006). It has been proposed that, if *S. suis* fails to induce acute fatal septicemia but the level of bacteremia remains high, CNS disease may appear in later stages of the infection (Gottschalk et al., 2007). A recent study of *S. suis* meningitis in mice showed that cells lining the choroid plexus and the brain endothelium are potential entry sites for this pathogen into the CNS (Dominguez-Punaro et al., 2007). In

addition, a previous study demonstrated the ability of *S. suis* to adhere to and invade immortalized porcine BMEC (Vanier et al., 2004). An important role in these two processes has been suggested for surface proteins of *S. suis* (Vanier et al., 2007). On the other hand, it is known that one of the major outcomes of cell envelope charge stabilization by LTA D-alanylation is the efficient display of proteinaceous adhesins and/or invasins (Neuhaus and Baddiley, 2003). Indeed, in a number of Gram positive pathogens it has been shown that absence of LTA D-alanylation impairs adherence to and invasion of various cell lines (Abachin et al., 2002; Kristian et al., 2005; Poyart et al., 2003). Our SCOTS results showed that expression of the *dlt* operon is upregulated upon interaction of *S. suis* with porcine BMEC (Article I). In Article II, we clearly demonstrate that the interactions of *S. suis* with these cells are impaired in the absence of LTA D-alanylation, perhaps as the consequence of inefficient display of putative adhesins/invasins in a more electronegative envelope surface. We speculate that this impaired interaction with porcine BMEC, together with failure of the Δ*dltA* mutant to maintain a high level of bacteremia, are responsible for the reduced ability of the Δ*dltA* mutant to induce meningitis. Indeed, experimental infection of mice at the intermediate dose of infection clearly demonstrated that the Δ*dltA* mutant was less fit to survive in blood and to induce CNS disease. On the other hand, it should be pointed out that absence of LTA D-alanylation did not prevent the development of meningitis in both swine and mice at the high dose of infection. This was not surprising, since levels of bacteremia were high for several days in inoculated animals and, although it was impaired in its interactions, the Δ*dltA* mutant was still able to invade porcine BMEC.

As stated above, many previous studies have shown impaired interaction of Δ*dltA* mutants with different cell lines (Abachin et al., 2002; Kristian et al., 2005; Poyart et al., 2003). However, an important novel finding of the present research project is the demonstration of reduced adhesion to and invasion of porcine BMEC by the *S. suis* Δ*dltA* mutant. In the characterization of a Δ*dltA* mutant of GBS using the neonatal mouse model, it has been shown that this strain was impaired in its ability to induce CNS disease and was hardly found in the brain of infected animals (Poyart et al., 2003). These findings were attributed by the authors to the decreased survival of the mutant in blood. In this study, we provide further and novel evidence. We provide further and novel evidence that in addition to increased blood clearance, impaired interactions with BMEC are important mechanisms that explain why Δ*dltA* mutants of meningitis-causing streptococcal species are less fit to induce this disease.

In summary for this section, *S. suis* LTA D-alanylation mediated by the *dlt* operon contributes phenotypically to resistance to CAMPs, likely through an increased net positive surface charge. It also enhances the resistance of *S. suis* to neutrophil killing as well as its capacity for adherence to and invasion of porcine BMEC. In addition, LTA D-alanylation contributes to *S. suis* virulence in both the murine and the porcine models of infection, probably through interference with innate immune clearance mechanisms and by facilitating penetration of host barriers. These results strongly suggest that LTA D-alanylation is an important virulence factor of *S. suis*.

Determination of *S. suis* peptidoglycan fine structure

Peptidoglycan is an essential component of the bacterial cell wall and, also, an important target for the innate immune system (Boneca, 2005; Chaput and Boneca, 2007). One of the most important and widespread compounds of the constitutive defense system is lysozyme, a muramidase that cleaves peptidoglycan between the glycosidic β-1, 4-linked residues of NeuNAc and GlcNAc. It is produced by various tissues (mucous membranes, lower respiratory tract or intestinal tract) and is present in body fluids, such as serum, saliva, sweat and tears (Bera et al., 2005). The lysozyme titer is increased by infection, and lysozyme is induced in numerous types of mammalian cells, including monocytes and macrophages as well as neutrophils (Keshav et al., 1991). An increase in lysozyme concentration in blood is an indicator for monocyte and granulocyte proliferation. Interestingly, it has been reported that patients suffering from meningitis present increased titers of lysozyme in their CSF (Klockars et al., 1978). Lysozyme is clearly an important defense compound of the host. Therefore, pathogenic bacteria that colonize the host over a longer period or that are able to cause chronic infections have developed mechanisms to evade or resist the lysozyme defense. One of the mechanisms used by pathogenic bacteria is N-deacetylation of their peptidoglycan (Boneca et al., 2007; Vollmer and Tomasz, 2000). Indeed, several studies have shown that N-deacetylated peptidoglycan is a poor substrate for lysozyme, and that the activity of lysozyme can be restored by chemical acetylation of the substrate (Amano et al., 1977; Boneca et al., 2007; Vollmer and Tomasz, 2000; Westmacott and Perkins, 1979).

Peptidoglycan N-deacetylation is not the only modification with an effect on the activity of lysozyme. For example, O-acetylation of MurNAc and the covalent linkage of other cell-wall

polymers such as teichoic acid also increase resistance to lysozyme (Bera, 2007; Bera et al., 2005). However, peptidoglycan modification by N-deacetylation seems to be important for at least two major Gram-positive human pathogens. Indeed, pneumococci lacking a functional *pgdA* gene became more sensitive to lysozyme in the stationary phase of growth (Vollmer and Tomasz, 2000). A second *S. pneumoniae pgdA* mutant exhibited significantly reduced virulence in the intraperitoneal mouse model when compared to the parental strain, indicating that PgdA is a putative virulence factor (Vollmer and Tomasz, 2002). In addition, it has been reported that a *pgdA* mutant strain of *L. monocytogenes* was impaired in its ability to induce disease in the murine model of infection and that the *pgdA* gene was required by this species to resist the host innate immune response mediated by lysozyme (Boneca et al., 2007).

As shown in Article I, *S. suis* upregulated *pgdA* (SSU1448) expression upon interaction with porcine BMEC. We therefore put forward the hypothesis that *S. suis*, through the action of the *pgdA* gene product, has the ability to modify its peptidoglycan by N-deacetylation. We reasoned that by modifying its peptidoglycan, *S. suis* might achieve resistance to the innate host immune response mediated by this enzyme. We therefore decided to carry out an assessment of the contribution of this putative modification to the virulence traits of *S. suis*. Functional analysis of *S. suis pgdA* function faced, however, an important drawback caused by the lack of knowledge on the composition and fine structure of *S. suis* peptidoglycan. Indeed, even though it has been proposed as an important factor for *S. suis* virulence (Higgins and Gottschalk, 2006), knowledge on the cell wall composition of this pathogen was very limited at the beginning of this research project. As discussed above, a partial structure of *S. suis* LTA had been reported (Elliott et al., 1977). However, the peptidoglycan of this bacterium had largely remained elusive. Therefore, we first carried out the determination of *S. suis* peptidoglycan fine structure. In fact, this research project describes for the first time the composition of this essential polymer in this important swine pathogen.

S. suis peptidoglycan was found to be, in several aspects, typical of species within the low G+C-content Gram-positive phylum *Lactobacillales*. Like in most other streptococci, in *S. suis* mostly D-Gln is found at position 2 of the stem peptide, and an L-Lys residue is present at position 3, which can participate in 4-3 cross-links with neighboring peptides (Schleifer and Kandler, 1972). However, unlike other streptococci, the peptidoglycan of *S. suis* lacks an interpeptide bridge but contains direct L-Lys-D-Ala cross-links. This direct link is common among

Gram negative bacteria but rare among Gram positive bacteria (Vollmer et al., 2008a). In fact, interpeptide bridges are found in the peptidoglycan of other human or animal streptococcal pathogens such as GAS (L-Lys-L-Ala_{2-3}), GBS [L-Lys-L-Ala_2 (L-Ser)], *S. bovis* [L-Lys-L-Thr-L-Ala (L-Ser)] or *Streptococcus uberis* (L-Lys-L-Ala_{3-4}) (Schleifer and Kandler, 1972). Consistent with the observed results, database searches revealed the absence in *S. suis* of homologues of the genes encoding for known peptide branching enzymes of the Fem transferase or ATP-grasp families (Bellais et al., 2006; Filipe and Tomasz, 2000) (data not shown). In *S. suis* about 67% of the peptides were found to be present in dimeric or trimeric cross-links [Table VIII, (Article III, Supplemental Table 1)]. *S. suis* peptidoglycan has a low proportion of pentapeptides and tetrapeptides indicating high activities of DD- and LD-carboxypeptidases in this species. Finally, some of the minor muropeptides correspond to modifications of canonical structures, suggesting the presence of glucosaminidase, amidase and N-deacetylase activities (Article III).

N-deacetylation of peptidoglycan and the virulence of *S. suis*

As mentioned above, in this research project we determined the muropeptide composition of *S. suis* peptidoglycan and found N-deacetylated compounds. Comparison with an isogenic Δ*pgdA* mutant showed that the product of the *pgdA* gene was responsible for this specific modification. As mentioned, some bacterial species resist lysozyme activity by means of peptidoglycan N-deacetylation and it has been proposed that the level of this modification may correlate with the level of resistance (Herbert, 2007; Vollmer, 2008). Interestingly, in the *S. suis* WT strain, peptidoglycan N-deacetylation occurred in very low amounts during growth in normal laboratory culture medium (Article III). Consistently, the WT strain was sensitive to lysozyme at concentrations much lower than those allowing growth of *S. pneumoniae* and *L. monocytogenes* (Boneca et al., 2007; Vollmer and Tomasz, 2000). Furthermore, no major differences between the *S. suis* WT and Δ*pgdA* mutant strains regarding resistance to the action of lysozyme *in vitro* were observed. In fact, these results may be explained by an already low degree (probably < to 1 % of total muropeptide) of peptidoglycan N-deacetylation of the WT strain. By comparison, resistance to lysozyme in *S. pneumoniae* correlated with over 80% of N-deacetylated glucosamine residues (Vollmer and Tomasz, 2000). In *L. monocytogenes* the extent of N-deacetylation was lower, but still very important, with up to 50 % of N-deacetylated muropeptides (Boneca et al., 2007). In this regard, this research project presents novel and interesting data. Indeed, only six reports on *pgdA* are currently available in the literature

(Boneca et al., 2007; Deng et al., 2008; Hebert et al., 2007; Meyrand et al., 2007; Vollmer and Tomasz, 2000, 2002).

So far, four other reports have shown that PgdA confers a high level of N-deacetylation for *S. pneumoniae*, *L. monocytogenes* and *L. lactis* when these species are grown *in vitro* (Boneca et al., 2007; Meyrand et al., 2007; Vollmer and Tomasz, 2000, 2002). Interestingly, the other two studies (Deng et al., 2008; Hebert et al., 2007) have questioned the link between expression of PgdA homologues and peptidoglycan N-deacetylation. In fact, *S. mutans* cells in which the *pgdA* gene had been deleted displayed a different colony texture and a slightly increased cell surface hydrophobicity, yet did not become hypersensitive to lysozyme. Moreover, recombinant *S. mutans* PgdA (highly homologous to the former two species PgdA) showed no activity towards shorter chitooligosaccharides or a synthetic peptidoglycan tetrasaccharide. The authors suggested that *S. mutans* PgdA may not act on peptidoglycan but, instead, on an as yet unidentified polysaccharide within the bacterial cell surface (Deng et al., 2008). In the same line, *E. faecalis* PgdA seems to lack peptidoglycan N-deacetylase activity, in spite of its high homology to *S. pneumoniae* and *L. monocytogenes* PgdA enzymes. Similar to *S. mutans*, no evidence for peptidoglycan *N*-deacetylase activity was found for the histidine-tagged recombinant *E. faecalis* protein when incubated directly with purified peptidoglycan (Hebert et al., 2007). These data are in agreement with the literature, since no N-deacetylated peptidoglycan has been reported for *E. faecalis*in previous studies (Hebert et al., 2007).

The two later articles relied on the use of recombinant proteins to disregard involvement of PgdA in peptidoglycan modification (Deng et al., 2008;Hebert et al., 2007). However, it is not uncommon that the level of activity of a recombinant protein does not reflect its activity in the cell. Indeed, unpublished work by Bui et al at the Vollmer's laboratory has shown that a soluble form of recombinant *S. pneumoniae* PgdA [the only peptidoglycan deacetylase in *S. pneumoniae* (Vollmer and Tomasz, 2000)] expressed in *E. coli* had only poor activity against isolated peptidoglycan or muropeptides, although the fraction of deacetylated muropeptides in WT *S. pneumoniae* cells is very important and the *S. pneumoniae pgdA* mutant strain did not contain any detectable amount of deacetylated muropeptides (Dr. Waldemar Vollmer, personal communication). In addition, these two reports have used the less sensitive HPLC-MALDI-TOF-MS to investigate the presence of N-deacetylated muropeptides. As shown in Article III for *S. suis*, this approach may result in false negative results.

Despite contradictory results in the literature regarding the role of *pgdA*, the *S. suis pgdA* gene product is clearly involved in peptidoglycan N-deacetylation. In this regard, the demonstration that *S. suis* upregulates the expression of the *pgdA* gene upon interaction with neutrophils as well as during growth *in vivo* (Article III) constitutes an interesting and unprecedented finding of this work. Since it is apparent that the *S. suis pgdA* gene product is responsible for peptidoglycan N-deacetylation, it is tempting to speculate that the increased expression of the *S. suis pgdA* gene *in vivo* correlates with a higher degree of PG N-deacetylation under these conditions that, in turn, results in enhanced resistance to lysozyme produced by neutrophils. However, in view of the fact that it is technically impossible to prepare peptidoglycan from these samples in amounts and purity suitable to perform HPLC and LTQ-FT mass spectrometry, we are unable to provide conclusive evidence for this hypothesis. A possible way to further assess this issue in future studies might be the complementation in trans of both the WT and mutant strain with the *S. suis pgdA* gene placed under the control of a strong constitutive promoter.

Results of the experimental infections carried out in this research project using the Δ*pgdA* mutant suggest that peptidoglycan N-deacetylation is a major virulence factor of *S. suis*. Overall, the infection outcome in the mouse clearly indicates that the Δ*pgdA* mutant is severely hampered in its ability to inflict damage to the host. The most important consequences of peptidoglycan N-deacetylation are increased survival of *S. suis* at early stages of the infectious process and an increased ability of the pathogen to survive in blood. Interestingly, there was a complete absence of septic shock signs in mice inoculated with the Δ*pgdA* mutant and the production of inflammatory mediators was completely abrogated in mice inoculated with the Δ*pgdA* mutant, while, in perfect agreement with a previous study (Dominguez-Punaro et al., 2007), an exacerbated Th1 inflammatory response in mice inoculated with the WT strain was observed. Different results were obtained in a previous study with *L. monocytogenes*. In fact, peptidoglycan purified from the WT *L. monocytogenes* poorly activated the NF-κB pathway leading to the production of various chemokines, growth factors, antimicrobial peptides, proinflammatory cytokines, and other products required for clearance of the infection. In comparison, fully acetylated peptidoglycan purified from the *pgdA* mutant increased NF-κB activation in a Nod1- and Nod2-dependent manner (Boneca et al., 2007). On the other hand, the *L. monocytogenes* study evaluated NF-κB activation using *in vitro* experiments and did not

actually investigate the production of cytokines in experimentally inoculated animals. Nevertheless, and although we have not yet tested this hypothesis, we consider it unlikely that the *S. suis* fully acetylated peptidoglycan has an impaired capacity to activate the NF-κB pathway. Instead, we propose that the observed basal levels of pro-inflammatory cytokines in mice inoculated with the Δ*pgdA* mutant might result from the rapid clearance of the mutant by neutrophils (low levels of bacteremia), along with an irrelevant activation of macrophages, lymphocytes and NK cells in this process.

In spite of the fact that the Δ*pgdA* mutant was able to induce clinical signs in some pigs, conclusions obtained for the mouse may be extended to the pig. Indeed, mortality observed in the Δ*pgdA* mutant group may be explained, at least in part, by the fact that we have prioritized the use of the highly aggressive intravenous route of administration and a high dose of inoculum in order to use a validated model of infection (Berthelot-Herault et al., 2001a). Nevertheless, in contrast to the WT group, pigs that survived septic shock in the Δ*pgdA* mutant group noticeably recovered, and it was apparent that the Δ*pgdA* mutant was less fit to survive in blood. This inability to persist in blood might also be important for later stages of the infection. Firstly, colonization of organs was hampered in the mutant group in both the mouse and pig. In addition, it has been shown that if *S. suis* fails to induce acute fatal septicemia but the level of bacteremia remains high, CNS disease may appear afterwards (Dominguez-Punaro et al., 2007; Segura et al., 2006). Interestingly, and in agreement with its observed rapid clearance from blood, we did not record any case of meningitis in either model with the Δ*pgdA* mutant. On the other hand, meningitis was responsible for the death of 35 % of mice and 10 % of pigs in the respective WT groups.

Enhanced clearance of the Δ*pgdA* mutant strain from blood might be attributed to an increased susceptibility of the mutant to killing by neutrophils, as showed in Article III. We speculate that differences observed in killing by neutrophils between strains might primarily be explained by the enhanced resistance of the WT strain to lysozyme secreted by neutrophils, due to increased peptidoglycan N-deacetylation after induction of the *pgdA* gene. However, it is possible that the effect of peptidoglycan N-deacetylation on the interactions of pathogens with the host might be multifactorial and not restricted to the effect of lysozyme activity. In fact, peptidoglycan N-deacetylation introduces additional positive charge into the cell wall, potentially affecting the binding of specific cell-wall proteins. As discussed for LTA D-alanylation,

increasing the positive charge of the cell wall is likely to increase the resistance of the bacterium to CAMPs. Thus, next to D-alanylation of LTAs (Article II), the N-deacetylation of *S. suis* peptidoglycan could be a second way to introduce positive charges into the cell wall to protect the pathogen against CAMPs of the host organism. Interestingly, and different from what was observed for the Δ*dltA* mutant, neither the WT or Δ*pgdA* mutant strains showed differences in resistance to the action of CAMPs such as polymixin B, magainin II, colistin and human neutrophil peptide (HNP)-1 and HNP-2, functional homologues of which are secreted by porcine neutrophils (Supplemental Table 2, Appendix). However, since the *pgdA* gene is upregulated *in vivo* by *S. suis*, it is possible that, *in vivo*, an eventual more N-deacetylated peptidoglycan might also result in a more positive cell wall. Therefore, in the absence of such a modification for the Δ*pgdA* mutant peptidoglycan, it could be proposed that in addition to lysozyme, CAMPs may also mediate killing of the mutant under *in vivo* conditions. However, a recent study in *S. pneumoniae* (whose peptidoglycan is highly N-deacetylated *in vitro*) reported no differences in sensitivity to human defensins HNP-1-3 between the WT strain and an isogenic encapsulated Δ*pgdA* mutant (Beiter et al., 2008). On the other hand, since our killing assay does not differentiate between intra and extracellular killing it is difficult to estimate if increased killing of the Δ*pgdA* mutant correlates with increased phagocytosis. However, since the Δ*pgdA* mutant is as encapsulated as the WT strain and, on the other hand, it has been shown that capsular material interferes with the uptake of *S. suis* by phagocytes (Chabot-Roy et al., 2006; Charland et al., 1998; Segura et al., 2004; Smith et al., 1999b), enhanced phagocytosis of the mutant might be unlikely. Indeed, we suggest an explanation similar to that put forward before for the *S. suis* Δ*dltA* mutant (Article II). Indeed since both Δ*pgdA* and Δ*dltA* mutants are encapsulated, they might be killed by porcine neutrophils extracellularly in NETs. Since neutrophils, in addition to delivering their complex antibiotic arsenal into the phagosome, also discharge their lysozyme-rich specific granules extracellularly in the NETs (Cho et al., 2005; Mollinedo et al., 2006) it might be postulated that the Δ*pgdA* mutant may also be killed through the action of this enzyme after being trapped in these extracellular structures. Further experiments are needed to confirm this hypothesis.

In conclusion for this section, we have determined the muropeptide composition of *S. suis* and showed that this bacterium, through the action of the *pgdA* gene product, modifies its peptidoglycan by means of N-deacetylation. We showed that this peptidoglycan modification,

which is probably enhanced *in vivo*, greatly contributes to the virulence of *S. suis* in both the murine and porcine models of infection. These results strongly suggest that PG N-deacetylation is also a major virulence factor of this swine pathogen and zoonotic agent.

V. CONCLUSIONS AND FUTURE DIRECTIONS

In this research project, in order to identify novel *S. suis* virulence factors, we have interrogated the differential gene expression of this pathogen during a key step of its pathogenesis of infection such as BBB crossing. Within the limits imposed by the use of an *in vitro* model of infection, the identification of the twenty eight SCOTS-identified genes reported here, which are preferentially expressed by *S. suis* upon its interaction with porcine BMEC, constitutes an important step forward for our comprehension of how this pathogen may cause disease. Indeed, some selected genes were characterized in this research project and shown to play important roles in *S. suis* pathogenesis of infection. Future characterization of the remaining SCOTS-identified genes will be of much importance for advancing our understanding on the mechanisms of *S. suis* disease development and progression. Besides, it might be anticipated that the current availability of *S. suis* whole genome sequence data will allow for SCOTS to be used in conjunction with DNA microarrays, which will further improve the deciphering of the molecular weapons *S. suis* makes use of for causing disease. Extending the analysis to identify transcriptional differences at different *in vivo* localizations (i.e., blood, brain, heart, tonsils) as well as at different stages of infection are possible future directions for further elucidation of *S. suis* virulence factors.

Evidence is accumulating that a variety of Gram positive surface proteins contribute to the virulence of these organisms. It seems likely, therefore, that *S. suis* surface proteins may play key roles during various stages of the infection. Particularly, it is apparent that proteinaceous adhesins/invasins mediate, at least in part, invasion of porcine BMEC. Indeed, results presented in this research project suggest that stabilization of surface net charges by D-alanylation of the LTA may be important for an efficient display and function of these putative adhesins/invasins. However, perhaps by the limitations imposed by the model used in this research project, an interesting outcome of this study was the lack of isolation of specific genes encoding *stricto sensu* adhesins and/or invasins. Indeed, the only putative structure that might have fulfilled that definition, the pilus encoded by cluster F, was in fact found to be adhesin-less and, as such, did not play a role in adhesion to or invasion of porcine BMEC nor was it essential for *S. suis* virulence in general, at least in the mouse. It is to expect that the wealth of information that is becoming available from genome sequences as well as the information about known surface proteins in other pathogenic Gram positive organisms will provide the

framework for future identification of *S. suis* surface proteins with adhesin and/or invasin capacities.

The ability to evade host immune surveillance is a critical virulence determinant for any pathogenic microorganism. Cell wall components such as the peptidoglycan and the LTA are often considered as static structures which provide supportive functions for maintaining and protecting the bacterial integrity. In this regard, this research project highlights that, in contrast to that view, these structures also fulfill a variety of virulence-related functions in *S. suis*. Indeed, both the peptidoglycan and the LTAs are actively modified by *S. suis* and these modifications constitute important immune evasion mechanisms that protect the pathogen at every stage of the infection. These contributions to virulence vary from resistance to host antimicrobial agents such as lysozyme and CAMPs, to killing by phagocytes, and to the ability to modulate the host inflammatory response. From the results presented here it is apparent that the *pgdA* and the *dlt* operon gene products, responsible for the modifications of these extracellular structures, are major *S. suis* virulence factors. However, this research project may be considered as the beginning of the characterization of the roles of LTA and peptidoglycan modifications in *S. suis*–host interactions. These extracellular structures deserve much more attention. In this regard, the study of the mechanisms which drive killing by neutrophils of well encapsulated *S. suis* strains in a background of absence of D-alanylation of the LTA and/or absence of N-deacetylation of the peptidoglycan is a potential future direction of this research project. In addition, the reported proinflammatory, endotoxin-like activities of the *S. suis* cell wall can now be further clarified by using the defined mutants generated in this research project.

VI. REFERENCES

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VII. APPENDIX

Pili and *S. suis* virulence

As discussed in the body of this thesis, a putative cluster encoding pili was identified in this research project by the use of SCOTS. Recently, we renamed the full cluster as pili cluster F (Takamatsu et al., 2008a) (Figure 31, Appendix). The class C sortase in the pilus cluster has been named SrtF, consistently with the nomenclature used in the past (Osaki et al., 2002). The two LPXTG proteins were named Sfp1 and Sfp2 (for *srtF*-associated pilin subunit) and, finally, the signal peptidase homologue was designated SipF (for signal peptidase in the *srtF* cluster) (Takamatsu et al., 2008a). Further, *in silico* analysis of the region showed that the deduced amino acid sequences of these four proteins show 29-60% identity with those of the corresponding genes in GBS PI-2b.

To demonstrate the expression of pili in *S. suis,* we decided to accomplish a full characterization of the *S. suis* pilus cluster F. In addition, we aimed at the evaluation of the role played by this pilus in *S. suis* pathogenesis of infection. To accomplish these objectives, we have generated precise, in-frame mutant strains for each of the four genes belonging to this pilus island (*sipF*, *sfp2*, *sfp2*, *srtF*) as well as the *srtA* gene encoding the housekeeping sortase SrtA in the well characterized *S. suis* serotype 2 virulent strain P1/7 (using a knock-out technology similar to that described in Articles II and III). In addition, using commercial vectors and standard procedures suitable for expression of proteins in *E. coli* we cloned, expressed and purified the LPXTG-proteins. Antibodies against these proteins were raised in rabbits and used for the identification of pili subunits by Western blot in the cell wall fraction of the WT and mutant strains. Finally, we tested the WT and the mutant strains generated above in our well-standardized model of invasion of porcine BMEC (Vanier et al., 2004) as well as *in vivo* in our murine model of infection (Dominguez-Punaro et al., 2007).

Results obtained so far suggest that *S. suis* expresses an adhesin-less F pilus which is composed of the major pilin subunit only. Indeed, Western blots of *S. suis* cell wall proteins revealed with rabbit anti-Sfp1 subunit antibodies (Figure 32, Appendix) clearly show, for the WT strain, the presence of polymers of the Sfp1 pilin subunit, compatible with a pilus structure in which this pilin subunit constitutes the backbone. Consistently, these structures are absent from the Δ*sfp1* mutant. The role of SrtF in pili polymerization is highlighted by the fact that only

monomers of Sfp1 are present on the cell wall of the corresponding Δ*srtF* mutant. These monomers are attached to the peptidoglycan through the action of SrtA, as shown by the fact that they are absent from the cell wall of the corresponding Δ*srtA* mutant (Figure 32, Appendix, Fittipaldi et al, manuscript in preparation). In this latter mutant, polymerized pilus structures are observed, but not the monomer, a phenotype that is compatible with the proposed transient non covalent anchoring of pili to the cell wall by the dedicated sortases in a strain lacking the housekeeping sortase (Budzik et al., 2007; Dramsi et al., 2006; Mandlik et al., 2008a). Interestingly, the *sipF* mutant was not affected in polymerization of the pilus backbone, which is different from previously reported results for GAS pili (Zahner and Scott, 2008). However, signal intensities of higher molecular weight polymers of Sfp1 seems to be weaker than that of the WT strain. The results may imply a yet undefined role for SipF in Sfp1 polymerization. On the other hand, we failed to observe pilus-compatible structures when revealing the blots with anti-Sfp2 antibodies (data not shown). Moreover, not even single monomers were revealed with these antibodies, suggesting absence of expression of the putative minor subunit of the pilus F cluster. These unexpected results are, however, consistent with new genome sequence data recently (May, 2008) released by the Sanger Institute (ftp://ftp.sanger.ac.uk/pub4/pathogens/ss). In a correction to previously released data, in addition to the four putative pilus-associated genes, a short ORF was annotated between *sipE* and *sfp2* (Figure 31, Appendix). This ORF and *sfp2* are currently deduced to form a single pseudogene (SSU0425) in the genome sequence data of *S. suis* P1/7 (Takamatsu et al., 2008a). When we sequenced the region in a large collection of serotype 2 strains, data showed that all of them presented the same nonsense mutation (Takamatsu et al., 2008a).

Despite these results, since a pilus comprised of a polymer of the major pilin subunit only is expressed by *S. suis*, we carried out an evaluation of its contribution to the adhesion and the invasion of porcine BMEC, using an *in vitro* model similar to that described in Article II. In addition, we assessed whether this pilus participates in the virulence of *S. suis* by experimental inoculation of CD 1 mice with the WT and mutant strains (details on this infection model are given in Articles II and III). Results showed that absence of expression of pilus F does not impair the interactions of *S. suis* with porcine BMEC nor does it affect virulence *in vivo* (Figures 33 and 34, Appendix, Fittipaldi et al, manuscript in preparation). Although we have not yet tested an

eventual contribution to the virulence of *S. suis* in the pig, which is the natural host of this pathogen, from the results presented above, it seems likely that the pilus encoded by cluster F is dispensable for adhesion to porcine BMEC and for *S. suis* virulence in general. Therefore, one may wonder why a gene member of this pilus was highly upregulated upon contact of the pathogen with porcine BMEC (Article I). Again, the concept of regulon may be important to answer this question. Indeed, it may be hypothesized that despite the fact that this pilus (remnant?) does not seem to fulfill a characterized function in *S. suis*, its expression is still triggered by (a) unknown signal(s) sensed by the pathogen upon contact with porcine BMEC.

In addition to pili cluster F genes, SCOTS identified the *srtE* gene (ssu0453), encoding one of the *S. suis* four previously described class C sortases (Dramsi et al., 2005; Osaki et al., 2002). Previous work on *S. suis* with serotype 2 reference strain S735 (Osaki et al., 2002), analysis of sequencing data from strains P1/7 and 89-1591, as well as sequencing of the region in the strain used for the SCOTS project, virulent isolate 31533 (Takamatsu et al., 2008a), indicated that the *srtE* gene is not flanked by genes encoding LPXTG proteins. In fact, in sequenced strain P1/7, a putative signal peptidase (SSU0450) is found in the vicinity of *srtE* (SSU0453), but the region lacks the major and the ancillary pilin subunits. Instead, a putative exported protein (SSU0451) and a transposase fragment (SSU0452) are found between the signal peptidase and *srtE* (unpublished data). It seems thus possible that the putative exported protein represents the N-terminal region of a pilus protein that has been truncated by a deletion event that generated the partial transposase sequence. Further analysis of a large collection of serotype 2 strains indicated that strains of this serotype do not possess a complete pili E cluster (Takamatsu et al., 2008a). Therefore, the functional significance of *srtE* upregulation upon contact with porcine BMEC remains obscure. Interestingly, we identified a putative pili cluster E upstream of *srtE* in serotype 1 reference strain NCTC 10237 (Figure 31, Appendix), but then again this pilus seems to be non functional (Takamatsu et al., 2008a). Indeed, although the predicted product of the third gene of the cluster (designated *sep1'*) has 35% identity with the N-terminal half of the fimbrial subunit-like protein SSA1633 of *Streptococcus sanguinis* SK36, no C-terminal sorting signal was found in Sep1'. However, BLASTX analysis revealed that the amino acid sequence translated from the downstream region of *sep1'* showed 40% identity with the C-terminal half of SSA1633, leading to the notion that *sep1'* of NCTC 10237 is interrupted by a

nonsense mutation (Takamatsu et al., 2008a). As for pilus cluster F in serotype 2 strains, the genetic organization of the pilus cluster E in serotype 1 strains is similar to that of pilus island 2b (PI-2b) of GBS COH1 (Takamatsu et al., 2008a; Telford et al., 2006) (Figure 31, Appendix).

On the other hand, a putative third pilus cluster has recently been described in strain P1/7 and in some Chinese isolates (Vanier et al., 2008a; Wang et al., 2008). We recently renamed this region as *S. suis srtBCD* cluster (Takamatsu et al., 2008a). It contains three sortase-like genes (*srtB*, *srtC*, and *srtD*) and four putative genes (designated *sbp1*, *sbp2*, *sbp3*, and *sbp4* [for *srtBCD*-associated pilin subunit]), which encode putative cell-wall anchor family proteins containing C-terminal sorting signals. Sbp3 and Sbp4 were partially similar to the putative collagen adhesion proteins of other Gram-positive bacteria, whereas Sbp1 had 34% identity with the cell-wall anchor family protein of GAS MGAS10750. However, *sbp2*, homologous to the major pilin subunit of the *rlrA* pilus island of *S. pneumoniae* TIGR4 (Telford et al., 2006) is truncated by a nonsense mutation in the sequenced strain P1/7 and in a large collection of serotype 2 strains, leading to the notion that no pilus structure can be expressed from this cluster in *S. suis* (Takamatsu et al., 2008a). On the other hand, a Tn*917* insertion in *sbp4*, which probably exerts polar effects in the downstream gene *sbp3*, has been found to substantially attenuate the virulence of the resulting mutant in both the murine and porcine models of infection (Vanier et al., 2008a). These results suggest that the minor subunits of the *srtBCD* pilus cluster might play a role in *S. suis* virulence even if an actual pilus is not formed.

From the data discussed above, it is apparent that at least highly virulents *S. suis* strains do not require the formation of pili structures for full virulence *in vivo*. This is an interesting finding, since a review of the literature shows that pili structures fulfill a variety of functions linked to virulence in other pathogenic streptococci, such as colonization of specific host tissues and the development of bacterial biofilms (Mandlik et al., 2008b). Moreover, a recent study with GBS has uncovered important functions of the pili backbone itself in pathogenesis and host immune responses (Maisey et al., 2008). In agreement, an *S. pneumoniae* mutant that lacks sortases and pilin subunits encoded by a single pilus gene cluster proved defective in binding to epithelial cells grown in cell culture (Barocchi et al., 2006). This nonpiliated mutant was attenuated in a mouse infection model and outcompeted by the WT strain in the upper airways,

lungs and blood. Importantly, piliated pneumococci evoked host inflammatory responses, reflected by elevated levels of TNF-α and IL-6 release compared with the deletion mutant. Inflammation might thus promote subsequent bacterial invasion of the tissue leading to damage to mucosal barriers (Barocchi et al., 2006). However, despite these findings, the notion that pili are essential for *S. pneumoniae* virulence has been challenged by a recent report which analyzed the prevalence of pili production by virulent strains of this species (Basset et al., 2007). From a total of 484 virulent strains tested, of which 361 were nasopharyngeal isolates and 123 invasive disease isolates, pili were present in only 21.4% of the strains. Moreover, there was no difference in the frequency of pili-positive strains in nasopharyngeal and bacteremic isolates. Taken together, these results suggest that despite the data obtained from mouse studies, pili do not in fact represent an important virulence factor for *S. pneumoniae* invasive disease in humans (Basset et al., 2007). It is to be expected that future epidemiological studies carried out with other streptococci will shed light on the actual contribution of pili to the virulence traits of pathogenic members of this important genus. So far, the latter *S. pneumoniae* report (Basset et al., 2007) sustains findings presented in this research project suggesting that these structures may be dispensable for *S. suis* virulence.

Supplemental figures

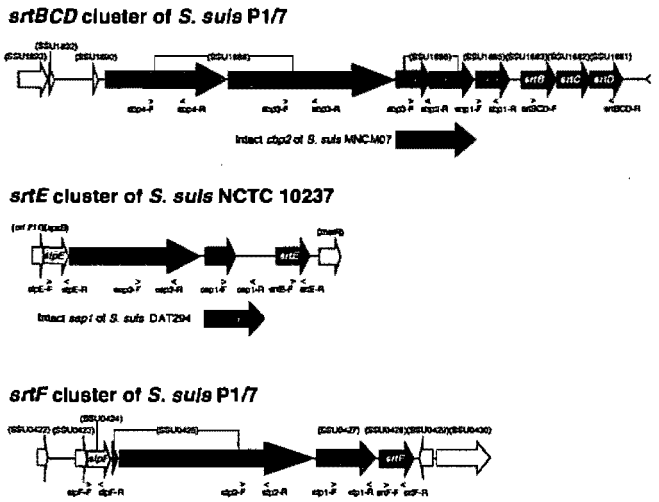

Figure 31. Genomic organization of the putative pilus gene clusters in *S. suis*

Black arrows, class C sortase genes; dark gray arrows, genes encoding putative pilin subunit proteins; light gray arrows, genes encoding signal peptidase homologues; open arrows and arrowheads outlined in grey, other genes. Gene *sfp2* and a short ORF upstream of it are deduced to form a single pseudogene (SSU0425), in the genome sequence data of *S. suis* P1/7 annotated by the Wellcome Trust Sanger Institute available at ftp://ftp.sanger.ac.uk/pub4/pathogens/ss. Adapted from Takamatsu et al. 2008a.

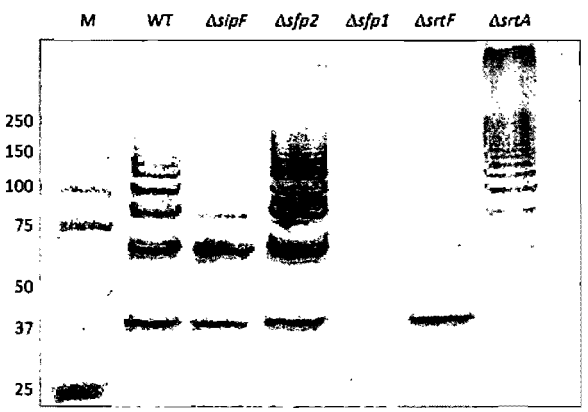

Figure 32. Detection of Sfp1 polymers by Western blotting.

Cell surface proteins were extracted from *S. suis* strain P1/7 and the mutant derivatives with mutanolysin, as described previously (Osaki et al, 2002). Proteins were separated by SDS-PAGE and then subjected to Western blot using polyclonal anti-Sfp1 sera. Lane 1, molecular weight markers. The anti-Sfp1 serum detected a ladder of high molecular weight polymers in P1/7 (lane 2). However, the ladder was not present in the Δ*sfp1* mutant (lane 5), suggesting that Sfp1 is polymerized on the bacterial surface. In the Δ*srtF* mutant, Sfp1 monomers, but not polymers were detected (lane 6), suggesting that SrtF is required for Sfp1 polymerization. In contrast, disruption of *sfp2* did not affect the polymerization of Sfp1 (lane 4). Interestingly, although Sfp1 polymers were detected in the Δ*sipF* mutant, signal intensity of higher molecular weight polymers of Sfp1 seems to be weaker than that of wild type strain. This result may imply an undefined role for SipF in Sfp1 polymerization. In the *srtA* mutant (lane 7), polymerized pilus structures are observed, but not the monomer, a phenotype compatible with the proposed transient non covalent anchoring of pili to the cell wall by the dedicated sortases in a housekeeping sortase-less genomic background. Adapted from Fittipaldi et al, manuscript in preparation.

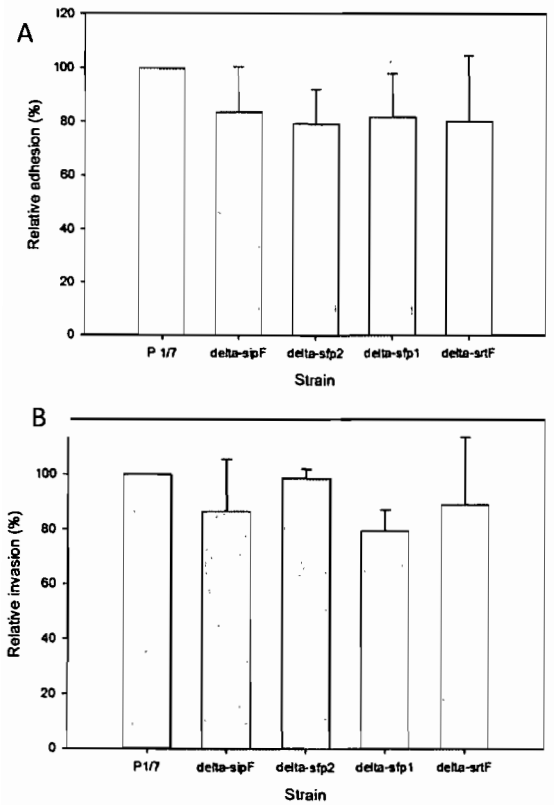

Figure 33. Interactions of the WT and pilus cluster F mutant strains with porcine BMEC.

The mutants showed no significant differences in levels of (A) adhesion to and (B) invasion of porcine BMEC. Data for the WT strain has been normalized to 100% Data are from at least 4 independent experiments. Adapted from Fittipaldi et al, manuscript in preparation).

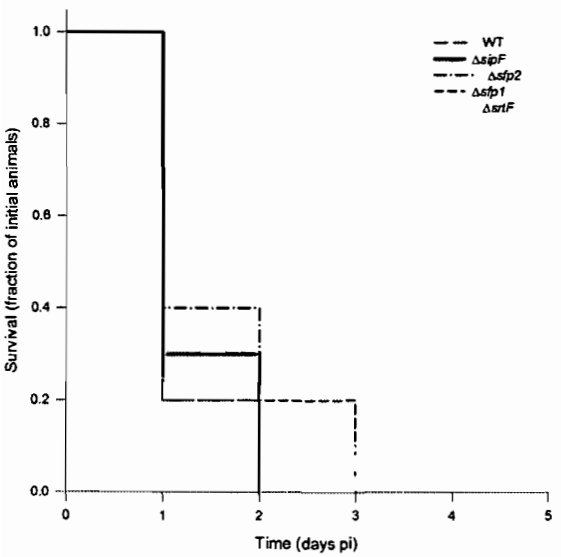

Figure 34. Survival of mice inoculated with the WT or the pili cluster F mutant strains.

No significant differences in survival were observed between groups (Log-Rank test, P>0.05)

Adapted from Fittipaldi et al, manuscript in preparation.

ARTICLE IV

"Potential use of an unencapsulated and aromatic amino acid-auxotrophic *Streptococcus suis* mutant as a live attenuated vaccine in swine."

Nahuel Fittipaldi, Josée Harel, Benoit D'Amours, Sonia Lacouture, Marylène Kobisch and Marcelo Gottschalk.

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Author contributions: NF, JH and MG designed research; NF, BDA, and SL performed research, MK contributed new reagents/analytic tools; NF, BDA, JH and MG analyzed data; NF wrote the manuscript and MG and JH revised the manuscript.

Although this article is part of, and describes research carried out during, the graduate studies of the candidate, the subject was not part of the original research plan for the candidate. Therefore, it is included in the Appendix section.

Details on the role of the candidate in the conception of the article: I contributed to research design, performed research, analyzed data and wrote the paper

Potential use of an unencapsulated and aromatic amino acid-auxotrophic Streptococcus suis mutant as a live attenuated vaccine in swine.

Nahuel Fittipaldi [a], Josée Harel [a], Benoit D'Amours [a], Sonia Lacouture [a], Marylène Kobisch [b] and Marcelo Gottschalk [a]*.

[a] Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP), Centre de Recherche en Infectiologie Porcine (CRIP) and Canadian Research Network on Swine Infectious Diseases. Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, J2S 7C6, Canada. [b] Agence Française de Sécurité Sanitaire des Aliments, Laboratoire d'Études et de Recherches Avicoles et Porcines, Unité de Mycoplasmologie-Bactériologie, 22440 Ploufragan, France

Running title: Live vaccine candidate against *Streptococcus suis* infections.

Keywords: *Streptococcus suis* ; *aro* operon ; attenuated vaccine candidate.

* Corresponding author. Mailing address: GREMIP, Faculté de Médecine Vétérinaire, 3200 rue Sicotte, CP 5000, St-Hyacinthe, Québec, J2S 7C6, Canada. Phone: (450) 773-8521, ext. 18374 Fax: (450) 778-8108. E-mail: [redacted]

Abstract

Streptococcus suis is responsible for severe economic losses to the swine industry. Prevention of the diseases caused by this pathogen is hampered by the inability of available vaccines to generate a protective response in pigs. A non-virulent, aromatic amino acid-auxotrophic and unencapsulated mutant strain of *S. suis* was generated in this study and a preliminary evaluation of its protective capacities was conducted in swine. Deletion of the cognate promoter of the *aro* operon in *S. suis* virulent strain S735 resulted in the abolishment of the expression of the four members of the operon, *aroA*, *aroK*, *pheA* and *orf10*. The resulting mutant strain BD101 was auxotrophic for aromatic amino acids as demonstrated by its failure to grow in a chemically defined medium unless it was supplemented with these compounds. In addition, as a result of the deletion of the cognate promoter of the *aro* operon, mutant BD101 lost its encapsulated phenotype. A protection assay was performed by immunisation of pigs with live strain BD101. Vaccination resulted in minor clinical signs but did not substantially impair the growth of vaccinated animals. Immunisation of animals with live mutant BD101 induced a considerable antibody response against *S. suis*. Vaccinated pigs presented only minor clinical signs and a survival rate of 100%, while 57 % of non-vaccinated animals died, after a challenge with the virulent parent strain S735. In order to prevent *S. suis* infections in swine, it may be useful to further evaluate strain BD101 as a vaccine candidate.

1) Introduction

Streptococcus suis is an important pathogen of swine causing meningitis, sudden death, septicaemia, endocarditis and pneumonia. Among the 35 described serotypes, serotype 2 is the most frequently associated with disease [1]. This serotype is also a zoonotic agent that has been identified as a cause of septicaemia, endocarditis, meningitis and toxic shock syndrome in humans [2-4]. Despite increasing research in recent years, knowledge on the pathogenesis of *S. suis* infections remains limited. Previous studies have reported on several *S. suis* putative virulence factors, including the polysaccharide capsule, the cell wall, and several proteins such as the extracellular factor (EF) protein, the muramidase-released protein (MRP) and a hemolysin known as suilysin [1]. The absence of one or more of these proteins, however, may not necessarily be associated with a lack of virulence, since MRP, EF and hemolysin-negative mutants of *S. suis* serotypes 1 and 2 have been shown to be as virulent as the respective wild-type strains after experimental infection of newborn, germ-free pigs [5, 6]. In addition, most virulent strains isolated in North America do not produce these proteins [7, 8]. On the other hand, the capsule of *S. suis* serotype 2 has proven to be critical for the virulence of the organism. Using unencapsulated isogenic mutants, it has been shown that the absence of a capsule correlated with increased phagocytosis by porcine macrophages and killing by porcine neutrophils [9-11]. Moreover, unencapsulated mutants have been shown to be impaired in virulence in two different porcine models of infection [9, 10].

No efficient vaccine against *S. suis* is currently available [1]. Several studies have reported protection of pigs using killed whole cells. However, the protection observed depended upon repeated immunization and/or was either serotype or strain dependent [12, 13]. Attempts to elicit protective immunity by vaccination with purified capsular polysaccharide have failed [14]. However, differences in protection between bacterins from a wild type virulent strain and its unencapsulated isogenic mutant (which was less protective) have raised the hypothesis that capsular polysaccharide is needed for full protection against homologous challenge [15]. Interest has recently shifted toward protein antigens of *S. suis* as vaccine candidates. Subunit vaccines using suilysin [16] or MRP and EF [17] have been shown to protect pigs from homologous and heterologous serotype 2 strains. However, since these proteins are

not widely present among virulent serotype 2 strains, their usefulness is limited. More recently, a preliminary evaluation of an *S. suis* surface protein (Sao protein) has shown that this protein is able to elicit a significant humoral response in immunised animals. However, the antibody response did not result in full protection of pigs that were intratracheally challenged with a virulent strain [18].

A live vaccine based on an avirulent strain and able to induce protective immunity may be of interest to counter *S. suis* infections in swine. We have previously obtained, using Tn*916* transposon mutagenesis, a *S. suis* serotype 2 unencapsulated non-virulent mutant strain (mutant 2A) [9]. In this work, we reproduced the unencapsulated phenotype of mutant 2A by allelic exchange and showed that the resulting mutant strain is affected in aromatic amino acids biosynthesis. We also describe the preliminary evaluation of this new mutant strain as a live vaccine in a protection assay in swine.

2) Materials and methods

2.1) Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study, along with their phenotypes and sources, are listed in Table 1. *S. suis* strains were grown in Todd-Hewitt broth (THB) or on Todd-Hewitt agar (THA), supplemented when required with 400 µg/ml kanamycin (Km) (Roche Diagnostics, Laval, QC, Canada). A chemically defined medium (CDM) allowing *S. suis* growth was adapted from the CDM medium formulated for other streptococci [19]. *E. coli* strains were grown on Luria-Bertani broth or agar containing, when needed, 40 µg/ml Km.

2.2) Nucleic acid techniques.

If not otherwise indicated, routine DNA manipulations were carried out as previously described [20]. Genomic DNA from *S. suis* and *E. coli* was prepared by the guanidium thiocyanate method [21]. Custom primers were purchased from Invitrogen (Invitrogen, Burlington, Ontario, Canada). Total RNA was prepared by using Trizol (Invitrogen), as recommended by the manufacturer. Northern and Southern-blots were performed using the DIG-labelling and detection kit (Roche), as recommended by the manufacturer. Restriction

enzymes, T4 DNA ligase, Taq DNA polymerase, calf intestinal alkaline phosphatase and the Klenow fragment of *E. coli* DNA polymerase I were purchased from Amersham (Amersham Biosciences, Piscataway, NJ). All other reagents and chemicals were purchased from Sigma (Sigma-Aldrich, Oakville, Ontario, Canada). PCR fragments were cloned using the TA Cloning Kit (Invitrogen), following the manufacturer's instructions.

2.3) Identification of the insertion site of Tn*916* in *S. suis* unencapsulated mutant 2A.

The insertion site of Tn*916* in *S. suis* mutant 2A was determined using a PCR-based procedure allowing amplification of one end of the Tn*916* insert and the adjacent genomic DNA sequence [22]. Mutant 2A genomic DNA was digested with *Hind*III, which cleaves the Tn*916* at a unique site. Resulting fragments were ligated into pBluescript KS+ (Stratagene, La Jolla, California, USA) previously digested with *Hind*III and treated with calf intestinal alkaline phosphatase. PCR was performed with primers Tn, specifically annealing to Tn*916*, and T7, annealing to the vector (Table 2). A region of 8.4 kb (Figure 1A) surrounding the Tn*916* insertion site was then sequenced using a primer walking strategy. DNA sequences were determined at the DNA Sequencing Facility of the University of Maine (Orono, Maine, USA), on a 373A DNA Sequencing System (Applied Biosystems, Foster City, California, USA). The BLAST software package was used to determine sequence homologies in the GenBank databases (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4) Single insertion mutant BD105 (*orf2*::pBEA1013).

A temperature sensitive suicide vector pBEA756 (Figure 2) was constructed and used to generate mutants in *S. suis*. This plasmid is a derivative of pCIV2 [23]. It carries a Km resistance element and an *E. coli colE1* origin of replication. A thermosensitive Gram positive replication origin (*repAts*) was PCR amplified from plasmid pVE6007 [24] using primers TS1F and TS1R, which introduce *Kpn*I restriction sites (Table 2). The resulting 1.5 kb fragment was cloned into the *Kpn*I site of pCIV2 to obtain pBEA756. The *orf2* gene was interrupted in *S. suis* virulent strain S735 by insertion of a recombinant plasmid. A 741 bp amplicon corresponding to an internal section of *orf2*, was amplified using primers B2F and B2R (Table 2), digested with the appropriate restriction enzymes and cloned into pBEA756 to obtain plasmid pBEA1013, which

was propagated in *E. coli*. Purified pBEA1013 was electroporated into *S. suis* as previously described [25]. Transformants were grown on Km-selective plates at 28°C (permissive temperature for the Gram positive replication origin). Insertion mutants were selected by shifting the temperature to 37°C (non-permissive temperature) in the presence of Km selection. Integration of pBEA1013 into the *S. suis* genome was verified by PCR and Southern blot.

2.5) Allelic replacement mutants.

Deletion of the promoter region of the *aro* operon was accomplished by allelic exchange using virulent strains S735 and 31533 (Figure 1C). Briefly, 2 PCR amplicons were obtained using primers pairs BA9 and BA7, and BA8G and BA11 respectively, which introduce *Bgl*II and *EcoR*I restriction sites (Table 2). *Bgl*II-digested amplicons were ligated to generate a mutant allele lacking the putative promoter and the 5' end of both *aroA* and *orf2*. The *EcoR*I-digested mutant allele was then cloned into pBEA756 to obtain plasmid pBEA860, which was propagated in *E.* coli. Plasmid pBEA860 was electroporated as previously described [25] into recipient *S. suis* strains S735 and 31533, which were plated on THA containing 400 µg/ml Km and incubated at 28°C for 48 h. Temperature was then shifted to 37°C to select for plasmid integration. Some single crossover integrants were then cultured several times at 28ºC in the absence of selection to obtain the excision and loss of the plasmid. Allelic exchange in Km sensitive clones was confirmed by PCR and Southern-blot.

2.6) Serotyping, coagglutination and biochemical identification.

Capsular reaction test, coagglutination and biochemical identification of wild-type and mutant strains were carried out as previously described [26]. The capsular sialic acid content was determined by the thiobarbituric acid assay [27].

2.7) Complementation studies in *S. suis* and *E. coli*.

Fragments spanning from the putative *aro* promoter to the end of *aroA* (primers AAK52 and ARA3EC), *aroK* (primers AAK52 and AAK3) and *orf10* (primers AAK52 and ORF10-4) were amplified by PCR, digested with the appropriate restriction enzymes and cloned into pBEA756 (Figure 1D). Recombinant resulting plasmids pBEAcomp1, pBEAcomp2 and pBEAcomp3 were

electroporated into *S. suis* mutant strain BD101. All the complementation studies in *S. suis* were carried out at 28°C. Restoration of capsule production in the resulting complemented mutants was assessed by dot blot with Z3 monoclonal antibody (Z3 mAb) directed against sialic acid [28], and by coagglutination with polyclonal serum directed against *S. suis* serotype 2 capsule. Complementation of *E. coli* AB2829 (*aroA*) was achieved using the inducible expression vector pMT020, a pACYC184 derivative that contains a chloramphenicol (Cm) resistance element, a *lac* promoter downstream of which lies the multiple cloning site and a *lacI* repressor gene upstream from the cloning site (M. Tessier, unpublished results). A 1.5 kb amplicon comprising the whole *aroA* gene of *S. suis* strain S735 was amplified using primers ARA3 and ARA5B and cloned into *Bgl*II and *Xba*I-digested pMT020, then electroporated into *E. coli* AB2829 strain. The transformants were spread on CDM agar plates containing Cm but lacking aromatic amino acids. IPTG was used as inducer of the *lac* promoter.

2.8) Transmission electron microscopy.

Electron microscopy was performed as previously described [29], with some modifications. Briefly, overnight (ON) cultures of *S. suis* wild type strain S735, mutant strain BD101 and complemented strain CA119 were mixed with specific anti-*S. suis* serotype 2 polyclonal serum and incubated at room temperature for 1 h. Cells were fixed in cacodylate buffer (0.1 M cacodylate, 5% v/v glutaraldehyde, 0.15% w/v ruthenium red, pH 7.2) for 2 h. After fixation, cells were immobilized in 4% (w/v) agar, washed in cacodylate buffer and post-fixed ON at 4°C in 2% (v/v) osmium tetraoxyde. Samples were dehydrated in graded series of ethanol and then embedded in Spurr low-viscosity resin. Thin sections were post-stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Model 420, Philips Electronics, The Netherlands).

2.9) Protection assay in pigs.

20 four-week old second generation of caesarean-deprived pigs (which tested negative for *S. suis* serotype 2 by ELISA [30] at the beginning of the experiment) were used in this study. All animals were treated according to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. Animals were divided in three groups: Group 1 (n=7) was

vaccinated with *S. suis* strain BD101. Groups 2 (*n*=7) and 3 (*n*=6) were not vaccinated. Strict biosecurity measures were implemented in order to avoid undesirable contamination of the pigs: an air filtration system and airlocks for each unit were used during the experiments. In addition, unit-specific clothes and showering before and after visiting the pigs were mandatory for researchers and technicians. Animals in group 1 received two doses of the vaccine (at 4 and 6 weeks old respectively) by intramuscular injection with a 2-ml, live mutant strain BD101 suspension at $1x10^9$ CFU/ml. After vaccination, fever, clinical symptoms, presence of *S. suis* in blood samples, daily food intake and weight gain were monitored. Animals from groups 1 and 2 were challenged 28 days after the first immunisation by intravenous injection of 10^9 CFU of *S. suis* strain S735. Group 3 were sham inoculated animals. The same parameters described above were monitored. After euthanasia (at day 42 after the first immunisation; 14 days after challenge) animals were examined for pathological lesions. Bacteriological isolation from blood and different organs was also performed. Briefly, samples weighing 0.5 g originating from the liver, the spleen, the lungs and the heart, were reduced to small pieces with a scalpel and diluted in 1 ml phosphate buffered saline (PBS), pH 7,3. Blood samples (100 µl) were also diluted in 1ml PBS. Sheep blood agar plates were inoculated with 100 µl of these suspensions and incubated ON at 37°C with 5% CO_2. In addition, an enrichment of the samples was performed by inoculation of 100 µl of blood or tissue suspensions in 5 ml THB and ON incubation at 37°C. Serial dilutions of enriched samples were then plated on sheep blood agar and grown as described above. In addition, articulations were swabbed and isolation was performed either directly using sheep blood agar, or after enrichment of the swab in THB, as described above. Isolated tiny α-hemolytic colonies were assigned to *S. suis* by serotyping as previously described [26]. Differences between groups regarding mortality, presence of lesions and *S. suis* isolation from blood and organs were assessed using the z-test for two proportions. Antibody response was verified in all animals by an indirect enzyme-linked immunosorbent assay (ELISA) using the whole unencapsulated mutant strain BD101 as antigen [30]. Sera originating from a pig immunised with strain S735 as well as from a healthy pig were used as positive and negative controls, respectively. Statistics was performed using the Student's t-test.

3) Results

3.1) Identification of Tn*916* insertion site in unencapsulated mutant 2A.

Tn*916* insertion site in *S. suis* mutant strain 2A was sequenced and characterised. The resulting 8.4 kb sequence has been deposited in GenBank, under accession number AY375303. The insertion site of Tn*916* in mutant 2A lies within the putative mRNA leader sequences of two divergent open reading frames (ORFs), *orf2* and *aroA*, transcribed in opposite directions (Figure 1A). Four other potential ORFs were deduced downstream of *aroA*. No function could be assigned to the ORF2 in a comparative search in the GenBank database. On the other hand, BLAST sequence comparisons indicated that the first three ORFs transcribed in the opposite direction are similar to *aroA*, *aroK* and *pheA* genes from *S. suis* and other Gram positive bacterial species (Table 3). Searches in public databases showed that the organisation of these 3 genes, which are putatively involved in aromatic amino acids biosynthesis, is almost identical in *S. suis* to that observed in several other streptococcal species. The *orf10* gene downstream *pheA* shares significant homologies with numerous transcriptional regulators of the LytR_cpsA_psr family, while the *orf13* is homologous to various RNA methyltransferases (Table 3). In Northern-blot experiments, a 4 kb mRNA was detected in the wild-type strain S735 using either *aroA* or *orf10* probes (data not shown). This result is consistent with a polycistronic mRNA spanning from the *aro* promoter to the end of *orf10*, which we will refer to as *aro* operon. In contrast, no mRNA hybridising with *aroA* or *orf10* probes could be detected in mutant 2A (data not shown). Thus, in mutant 2A transposon Tn*916* prematurely interrupts the transcription of the *aro* operon, exerting polar effects in the expression of genes *aroA*, *aroK*, *pheA* and *orf10*.

3.2) Inactivation of the *orf2*.

To evaluate if it was responsible for the abolishment of the unencapsulated phenotype, the *orf2* was interrupted by insertion of a recombinant plasmid in wild type strain S735. The resulting single crossover mutant BD105 (*orf2*::pBEA1013) (Figure 1B) reacted as the WT type strain in coagglutination tests with a polyclonal serum directed against *S. suis* serotype 2 capsule and with Z3 anti-sialic acid monoclonal antibody (Table 4), indicating that inactivation of the *orf2* does not affect capsule production.

3.3) Reproduction of the unencapsulated phenotype by allelic exchange.

Since the *orf2* does not play a role in capsule production, the unencapsulated phenotype of mutant 2A could be due to polar effects exerted by the insertion of Tn*916* on genes belonging to the *aro* operon. In order to reproduce the unencapsulated phenotype of mutant 2A in a stable mutant strain, the region comprising the *aro* putative promoter in strain S735 was deleted by allelic replacement, giving rise to strain BD101 (Figure 1C). In Northern-blot experiments no mRNA was found to hybridise to either *aroA* or *orf10* probes confirming that deletion of the *aro* promoter results in the abolishment of the expression of the entire *aro* operon in mutant strain BD101 (data not shown). Similar to mutant 2A, strain BD101 did not react in dot blot experiments with Z3 anti-sialic acid mAb. It was also negative in coagglutination tests with a polyclonal serum directed against *S. suis* serotype 2 capsule. Furthermore, dosage of capsular sialic acid content showed that this sugar was almost absent from strain BD101 (Table 4). The absence of capsule in mutant strain BD101 was also verified by electron microscopy (Figure 3). To further confirm that deletion of the promoter region of the *aro* operon abolishes capsule production in *S. suis*, an independent serotype 2 strain (virulent field strain 31533) was also mutated using the same strategy. The characteristics of the resulting mutant strain BD102 (absence of capsule by dot blot with Z3 mAb, sialic acid dosage and coagglutination) were similar to those obtained for mutant BD101 (Table 4).

3.4) Complementation of *S. suis* capsular function.

Complementation of capsule production was achieved by reintroducing different fragments of the *aro* operon into strain BD101 (Figure 1D). In each case, genes were under the control of their native promoter. Complementation with fragments comprising the region from the *aro* promoter to the end of *aroA* as well as from the *aro* promoter to the end of *aroK* failed to restore the capsulated phenotype. On the other hand, complementation with the entire *aro* operon (from the *aro* promoter to the end of the *orf10*) restored capsule production. Resulting strain CA119 reacted in dot blotting experiments with Z3 anti-sialic acid mAb and with polyclonal serum directed against *S. suis* serotype 2 capsule. However, complementation with the entire *aro* operon only partially restored capsule production (Table 4 and Figure 3).

3.5) Role of *S. suis aro* operon in aromatic amino acid biosynthesis.

Based on homology of functions, deletion of the promoter region of the *aro* operon should result in auxotrophy for aromatic amino acids. To test this hypothesis, we used a CDM allowing the growth of *S. suis* [19]. The wild-type *S. suis* strain S735 did not require the addition of aromatic amino acids to the CDM formulation to grow while mutant strain BD101 did not grow unless the medium was supplemented with these compounds. Complemented strain CA119 grew in both formulations (Table 5). To further confirm the involvement of the *S. suis aro* operon in aromatic amino acids biosynthesis, the *aroA* deficient *E. coli* strain AB2829 (*aroA*) was transformed with the expression plasmid pMT020 carrying the *S. suis aroA* gene. The complemented strain grew in CDM media lacking aromatic amino acids, while *E. coli* AB2829 (*aroA*) strain did not. The *E. coli* strain JM109, used as control, grew in any CDM formulation (Table 5).

3.6) Protection assays in pigs.

3.6.1) Vaccination:

After the first vaccine dose with mutant strain BD101, all animals in vaccinated group 1 presented slight hyperthermia 4 h post vaccination. Slight hyperthermia was also observed in 4 animals of this group after the second immunisation. Body temperature returned to normal values in all vaccinated piglets within 24 h. Vaccination did not induce any other symptoms. *S. suis* mutant strain BD101 was isolated in low counts from the blood of 4 pigs after the first immunisation and from 2 animals after the second immunisation. In most cases, an enrichment of the blood sample was necessary to isolate the strain. Until challenge, vaccinated animals showed growth and daily weight gain values similar to those in the non-vaccinated and control groups 2 and 3 (Table 6).

3.6.2) Experimental challenge:

Tenty-four hours after the experimental challenge with *S. suis* strain S735, hyperthermia was observed in all pigs belonging to vaccinated and non-vaccinated groups 1 and 2. However, average temperature was higher in non-vaccinated group 2. Two days after the challenge, only 3

vaccinated and all non-vaccinated pigs still presented hyperthermia. Lameness was observed in all pigs in group 1 from day 6 after challenge until euthanasia (Table 7). Articulations were hot and swollen at palpation. These latter symptoms were only punctual and did not persist over time. No mortality was observed in animals from group 1. On the other hand, lameness was observed in all pigs belonging to non-vaccinated group 2 from day 1 post challenge. Articulations were soaring during the 2 weeks that followed the challenge. Four out of 7 animals in group 2 were shaking 24 hours post challenge, and one pig showed convulsions. Four pigs died or were killed for humanitarian reasons in this group. *S. suis* serotype 2 could be isolated from the blood of all vaccinated and non vaccinated pigs. However, pigs in non-vaccinated group 2 showed higher bacterial counts than those observed in vaccinated group 1 (Table 7). Vaccinated animals presented higher growth and daily weight gain values compared to those of non-vaccinated group 2 during the 2 weeks following the challenge. However, after challenge, daily weight gain in vaccinated animals was slightly lower than in the sham-inoculated control group (Table 6). After the second immunisation, an increase in antibody titres was observed for all animals in vaccinated group 1. A few days after challenge, production of antibodies was observed in vaccinated group 1 and non vaccinated group 2. However, the effect was faster in vaccinated group 1 (Figure 4). Surviving pigs of all 3 groups were humanely killed two weeks post challenge. In vaccinated group 1, only articulations showed inflammation, with fibrin deposits and excess of synovial liquid. Macroscopic lesions were more common in non-vaccinated group 2, especially at the pleura, the pericardium and the peritoneum (Table 7). Fibrin deposits were observed in the liver and the spleen of all animals belonging to group 2. Furthermore, pneumonia and fibrinal pleurisy were observed for some animals of this group, while 5 animals also presented pericarditis. Bacterial isolation from different organs revealed a high bacterial colonization of the thoracic cage and abdominal organs. In vaccinated animals, bacterial isolation from organs was also possible, but to a much lesser extent (Table 7).

4) Discussion

In our laboratory we have previously obtained an unencapsulated *S. suis* serotype 2 mutant (mutant 2A) by means of transposon Tn*916* mutagenesis of *S. suis* virulent strain S735 [9]. In this study, we identified and characterised the insertion site of Tn*916* in mutant 2A.

Tn*916* was found to be inserted in the leader section of the mRNA of two divergent ORFs, *orf2* and *aroA*, this latter being the first gene of an operon also composed by *aroK, pheA* and *orf10*. No putative function could be assigned to the *orf2* after an exhaustive search in public databases. On the other hand, the 3 first genes of the *S. suis aro* operon, *aroA*, *aroK* and *pheA*, are similar to those of many other Gram positive and Gram negative bacteria. The *E. coli* counterparts of AroA and AroK play well established roles in the chorismate pathway, while PheA is a terminal enzyme of the aromatic amino acid biosynthesis pathway that catalyzes the conversion of prephenate to phenylpyruvate [31]. Chorismate is a common substrate in the biosynthetic pathways of aromatic amino acids and many other compounds like folate, ubiquinone and vitamin K [31]. In this work, we provide for the first time evidence that genes in the *S. suis aro* operon are indeed involved in the chorismate pathway. In fact, *S. suis aroA* complemented the *aroA* mutation in *E. coli* AB2829 (*aroA*), suggesting that the *S. suis* and *E. coli* chorismate biosynthetic pathways are, at least in part, similar. In addition, deletion of the promoter region of the *aro* operon in *S. suis* resulted in auxotrophy for aromatic amino acids as shown by the inability of the *S. suis* mutant strain BD101 to grow in CDM not supplemented with aromatic amino acids.

Capsular polysaccharide biosynthesis in *S. suis* has not yet been fully elucidated. In two subsequent studies the *S. suis* serotype 2 capsular locus (*cps2*) has been described and characterised [10, 32]. It has also been shown that nonencapsulated isogenic mutants can be generated by disruption of either the *cps2B* and *cps2EF* genes [10]. In this study we show that inactivation of the *aro* operon in both *S. suis* serotype 2 reference strain S735 and field strain 31533 results in an unencapsulated phenotype. Furthermore, complementation of mutant BD101 with a recombinant plasmid containing the entire *aro* operon allowed restoration, although partial, of the capsular phenotype. To the best of our knowledge, this is the first time a mutation in a region outside the *cps* locus resulting in abolishment of capsule production is described in *S. suis*. Although data presented in this study is insufficient to provide a full explanation for the absence of capsule in *aro* deficient mutants, it might be possible to hypothesize that capsule synthesis, transport or assembly production depend on the presence of chorismate-derived compounds. However, attempts to restore the unencapsulated phenotype of mutant BD101 by complementation with the *aroA* gene only or with both *aroA*

and *aroK* were unsuccessful. A role for *pheA* in capsule production could be difficult to be put forward; PheA is a regulatory enzyme involved in l-phenylalanine biosynthesis whose disruption should only cause an auxotrophy for this amino acid with, *a priori*, no major changes in global metabolism [31]. However, *S. suis* mutant strain BD101 growing in CDM medium containing phenylalanine did not revert to an encapsulated phenotype. On the other hand, the *orf10*, the last gene of the *aro* operon, is predicted to belong to the LytR_cpsA_psr family of transcriptional regulators. Interestingly, members of this family have been shown to act as positive regulators of capsule expression in both *Streptococcus agalactiae* and *Streptococcus pneumoniae* [33, 34]. It is therefore tempting to speculate that the *orf10* gene product might contribute to the regulation of capsule production in *S. suis* serotype 2.

Attempts to counter *S. suis* infections are hampered by the lack of efficient vaccines which can control the diversity of strains that cause disease [1].Vaccination with a live, attenuated strain of *S. suis* is one of the several approaches that may be used to elicit protective immunity in swine against this bacterium. Unencapsulated *S. suis* mutants have proven to be non-virulent for swine [9, 10]. Mutant strain BD101 is unencapsulated, and, besides, our results show that inactivation of the *aro* operon results in auxotrophy of this mutant for aromatic amino acids. Mutant strains in *aro* genes have been obtained in several bacterial species. Most of these mutants have proven non-virulent to their cognate hosts, since compounds derived from the chorismate pathway are essential for bacteria and not easily available in vertebrate tissues [35]. Furthermore, it has been shown using different animal models that protection against a challenge with a virulent homologous strain may be obtained following immunisation with *aro* mutants [36-39]. Since *S. suis* is also a zoonotic agent, safety of a live vaccine is of main concern, especially after the report of serious cases of human disease with a high rate of mortality described in China, that were directly linked to a concurrent outbreak of *S. suis* infection in pigs [40]. Based on the absence of capsule and auxotrophy for aromatic amino acids, mutant strain BD101 might be safely attenuated and, therefore, a good candidate for live vaccination of swine. Although we cannot completely exclude the possibility that the strain BD101 is not completely attenuated in humans, it is not virulent in the mouse model of infection (unpublished results), and, besides, results obtained in this study by monitoring the response to vaccination indicate that mutant strain BD101 is indeed of low virulence for swine. In addition, it

does not significantly affect animal growth. In fact, the only symptom observed after immunisation of animals was slight hyperthermia that subsided after 24 hours. After challenge with the homologous wild type virulent strain S735, the mortality in the vaccinated group was significantly reduced in comparison to the non-vaccinated group. As well, vaccinated animals clearly presented lower rates of clinical signs and no lesions characteristic of *S. suis* could be found on the brain, the liver, the spleen, the lungs and the heart of vaccinated animals.

Among *S. suis* serotypes, serotype 2 is responsible for most cases of disease in both swine and humans [1, 3]. Almost all studies on virulence factors, pathogenesis of the infection and vaccination have been carried out with this serotype [1, 41]. A previous study used a live, non-virulent, unencapsulated Δ*cpsEF* mutant as live vaccine. In that work, immunisation with the unencapsulated mutant strain failed to provide protection to pigs against the homologous serotype 2 parent strain [15]. By contrast, vaccination with mutant strain BD101 provided a relatively good protection level against a challenge with the homologous serotype 2 parent strain. Even if the mutant strains used for vaccination in that and the current studies are both unencapsulated and non-virulent for swine, other differences exist between the parent strains regarding both the genotype and phenotype. In addition, and perhaps more importantly, two different experimental models of infection were used in both studies [15, 42]. To evaluate virulence, different research groups have used different animal species, pigs with different health status and different ages, different routes of infection, or variable bacterial doses [43]. This fact has led to a controversy in the definition of "virulent" serotype 2 strains [43] and, actually, has hampered the studies on cross protection, because assessing the heterologous protection requires a set of strains whose virulence had been characterised in the same infection model. Recently, however, a standardised swine infection model for *S. suis* has been reported and used to compare the virulence of some field isolates of *S. suis* serotype 2 [42]. As new data regarding the level of virulence of different serotype 2 strains becomes available, it would be interesting to evaluate the ability of mutant strain BD101 to confer protection against heterologous virulent serotype 2 strains.

The actual contribution of humoral immunity to protection of pigs against *S. suis* is not fully understood [18]. Convalescent (and protected) animals do not present high antibody titres

against this bacterium (our unpublished observations). In the present study, the immunisation of pigs with live mutant BD101 did not elicit a vigorous humoral response after the immunisations. However, an acceptable level of protection against the challenge with the virulent homologous strain was observed. Host protection against infection caused by *S. suis*, a highly encapsulated microorganism, is mediated primarily by phagocytosis [9, 28]. For phagocytosis to occur efficiently the presence of opsonising antibodies capable of triggering leukocyte effector functions seems to be required [18]. On the other hand, it has been suggested that the generation of opsonising antibodies may depend on a Th1-like response [44]. It is thus tempting to speculate that vaccination with mutant strain BD101 might elicit a Type 1 immune response which in turn would drive the differentiation and activation of B cells to secrete antibodies with opsonising capacity. Further studies are needed to evaluate the contribution of humoral and cellular immunity in protection of animals against *S. suis* following vaccination with live mutant strain BD101.

The protection obtained by immunisation with mutant strain BD101 was not complete. Indeed, after the challenge, minor secondary effects were recorded among immunised animals. The absence of antibodies against capsular polysaccharide might be, at least in part, responsible for the inability of the BD101 vaccine to induce full protection in swine [15]. Secondary effects might also have been caused, at least in part, by the high dose of *S. suis* used for inoculation. As well, the extremely aggressive intravenous route of administration may have influenced the clinical onset observed in vaccinated animals. For *S. suis,* very few experimental infection models are available and most of them lack repeatability [43]. For that reason, this study has prioritised the use of a well-standardized infection model [42], in spite of the fact that it does not follow the natural route of infection of *S. suis* strains. Further studies using a different route of inoculation might help to better characterise the protective capacities of a live vaccine based on *S. suis* mutant strain BD101.

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Article IV Tables

Table XII. Article IV, Table 1. Bacterial strains and plasmids used in this study.

	Description	Source
Bacterial strains		
E. coli JM109	Host for cloning vectors. *endA1, recA1, gyrA96, thi, hsdR17* (r_k–, m_k+), *relA1, supE44,* Δ(*lac-proAB*), [F ′, *traD36, proAB, laqIqZ* Δ *M15*]	Promega
E. coli AB2829	AroA deficient strain. *glnV42* (AS), λ-, *aroA354*	CGSC [a]
S. suis S735	Serotype 2 (Reference strain; virulent)	Laboratory collection
S. suis 31533	Serotype 2 (Field strain; virulent)	Laboratory collection
S. suis 2A	Unencapsulated Tn*916* insertional mutant derived from strain S735. Tet^R.	Charland et al. [9]
S. suis BD101	Derived from strain S735. Deletion of the *aro* promoter resulting in abolishment of *aroA*, *aroK*, *pheA* and *orf10* expression	This work
S. suis BD102	Derived from strain 31533. Deletion of the *aro* promoter resulting in abolishment of *aroA*, *aroK*, *pheA* and *orf10* expression	This work
S. suis BD105	*orf2*::pBEA1013	This work
S. suis CA119	Mutant BD101complemented with the entire *aro* operon	This work
Plasmids		
pCR2.1	*E. coli* vector for cloning of PCR fragments	Invitrogen
pMT020	*E. coli* inducible expression vector	M. Tessier (unpublished)
pClV2	pUC *ori*, Km^R	Okada et al. [23]
pVE6007	Thermosensitive suicide vector (source of Gram positive *repAts*)	Maguin et al. [24]
pBEA756	Thermosensitive suicide vector for *S. suis* mutagenesis	This work
pBEA1013	pBEA756 carrying 761 bp internal sequence of the *orf2*	This work
pBEA860	pBEA756 carrying the construction for allelic exchange	This work
pBEAcomp1	pBEA756 carrying a fragment from the *aro* promoter	This work

	to the end of *aroA*	
pBEAcomp2	pBEA756 carrying a fragment from the *aro* promoter to the end of *aroK*	This work
pBEAcomp3	pBEA756 carrying a fragment from the *aro* promoter to the end of the *orf10*.	This work

[a] *Escherichia coli* Genetic Stock Center.

Table XIII. Article IV, Table 2. Oligonucleotide primers used in this study. Bold characters indicate restriction sites.

Primer name	Sequence (5'-3')	Restriction enzyme
Tn	CCACTTCTGACAGC**T**AAGAC	
T7	GTAATACGACTCACTATAGGGC	
TS1F	GTTA**GGTACC**GAAAGCCGATG	*Kpn*I
TS1R	TTGT**GGTACC**TAATTCAACTTCC	*Kpn*I
B2F	TCAC**GAATTC**TATATATCCCGTTCG	*Eco*RI
B2R	**T**GAA**GAATTC**AGGAAGGACTGATAGC	*Eco*RI
BA7	ATTG**AGATCT**TGTCTTGTCAACC	*Bgl*II
BA9	ACTC**GAATTC**TACGATGACCG	*Eco*RI
BA8G	ACTT**AGATCT**TATATCCCGTTCG	*Bgl*II
BA11	TTTG**GAATTC**ATTACCTAAAGTATC	*Eco*RI
AAK52	CACT**CTGCAG**CCACTCCCAATGTTG	*Pst*I
ARA3EC	GACT**GAATTC**GTCAGCAATAGGC	*Eco*RI
AAK3	CGTT**CTGCAG**GCCTTGATAACCTCTG	*Pst*I
ORF10-4	TTTCT**GAATTC**AGGCCTAACTC	*Eco*RI
ARA3	GACT**TCTAGA**GTCAGCAATAGGC	*Xba*I
ARA5B	ATTG**AGATCT**CACAAGTAATTGTC	*Bgl*II

Table XIV. Article IV, Table 3. Characteristics of the putative open reading frames (ORFs) within the 8.4 kb fragment containing the *S. suis aro* operon

ORF	Putative function (organism)	Gene	GenBank Access Nº
ORF2	hypothetical protein (*S. suis* 89-1591)		ZP_00874823
AroA	3-phosphoshikimate 1-carboxyvinyltransferase (*S. suis* 89-1591)	*aroA*	ZP_00874822
AroK	Shikimate kinase (*S. suis* 89-1591)	*aroK*	ZP_00874821
PheA	Prephenate dehydratase (*S. suis* 89-1591)	*pheA*	ZP_00874820
ORF10	Transcriptional regulator (*S. suis* 89-1591)		ZP_00874819
ORF13	tRNA (uracil-5-)-methyltransferase (*S. suis* 89-1591)	*trmA*	ZP_00874818

Table XV. Article IV, Table 4. Results obtained for capsule, sialic acid production and coagglutination by *S. suis* wild type and mutant strains used in this study

Bacterial strains	Coagglutination [a]	Dot blot [b]	Sialic acid content (µg/ml) [c]
S. suis S735	+[d]	+	4.83 ± 0.97
S. suis 31533	+	+	4.23 ± 0.26
S. suis 2A	-[e]	-	0.12 ± 0.03
S. suis BD101	-	-	0.06 ± 0.03
S. suis BD102	-	-	0.09 ± 0.02
S. suis CA119	+	+	2.20 ± 0.23
S. suis BD105	+	+	4.18± 1.02

[a] Evaluated as previously described [26] using polyclonal serum against *S. suis* serotype 2 capsule.

[b] Determined using the Z3 monoclonal antibody directed against sialic acid [28].

[c] Measured using the thiobarbituric acid method [27].

[d] Positive reaction.

[e] Negative reaction.

Table XVI. Article IV, Table 5. Growth of different *S. suis* and *E. coli* strains in a chemical defined medium (CDM) with and without aromatic amino acids (aaa).

Bacterial strains	CDM	CDM without aaa
S. suis S735	Normal[a]	Normal
S. suis BD101	Normal	None
S. suis BD102	Normal	None
S. suis CA119	Normal	Normal
E. coli JM109	Normal[b]	Normal
E. coli AB2829 *aroA354*	Normal	None
E. coli AB2829 complemented with *S. suis aroA*	Normal	Normal

[a] Similar to that observed on Todd-Hewitt broth

[b] Similar to that observed in Luria-Bertani broth

Table XVII. Article IV, Table 6. Daily weight gain in pigs (g).

Time (days)	Group 1 (n=7) Vaccinated	Group 2 (n = 7) Not-vaccinated	Group 3 (n = 6) Control
Before first immunization			
- 4 to 0	450	400	375
After first immunization			
0-3	600	633	666
3-7	575	725	675
7-11	400	675	525
11-14	1033	866	1033
After second immunization			
14-18	725	750	800
18-21	666	800	700
21-25	725	625	700
25-28	866	966	833
After challenge			
28-32	-225	-500	775
32-35	400	466 [a]	733
35-39	1125	300 [a]	875
39-42	866	890 [b]	800
28-39	549	43 [a]	804

[a] Data from 5 animals
[b] Data from 3 animals

Table XVIII. Article IV, Table 7. Effects of challenging the different groups of animals used in this study with *S. suis* virulent strain S735.

	Group 1 (n = 7)	Group 2 (n = 7)	Group 3 (n = 6)
	Vaccinated	Not-vaccinated	Control
Mortality [a]	0 [b]	4 [b]	0
Clinical symptoms			
-Lameness	+	+++	-
-Shaking	+	+++	-
-Convulsions	0	1	0
Lesions [c]			
-Brain	0	3	0
-Other organs [d]	0 [b]	6 [b]	0
-Articulations	3	4	0
Bacterial isolation			
-Articulations	4	7	0
-Organs [e]	1 [b]	6 [b]	0
-Blood	7 (86) [f]	7 (2000) [f]	0

[a] Natural mortality or euthanasia for humanitarian reasons.
[b] Significant differences between vaccinated and non vaccinated animals (z-test ; $P<0.05$).
[c] Macroscopic lesions characteristic of *S. suis* infection.
[d] The liver, the spleen, the lungs and the heart were examined.
[e] Isolation was performed from the same organs described in [c] and the brain.
[f] Mean bacterial counts (CFU/ml).

Article IV Figures

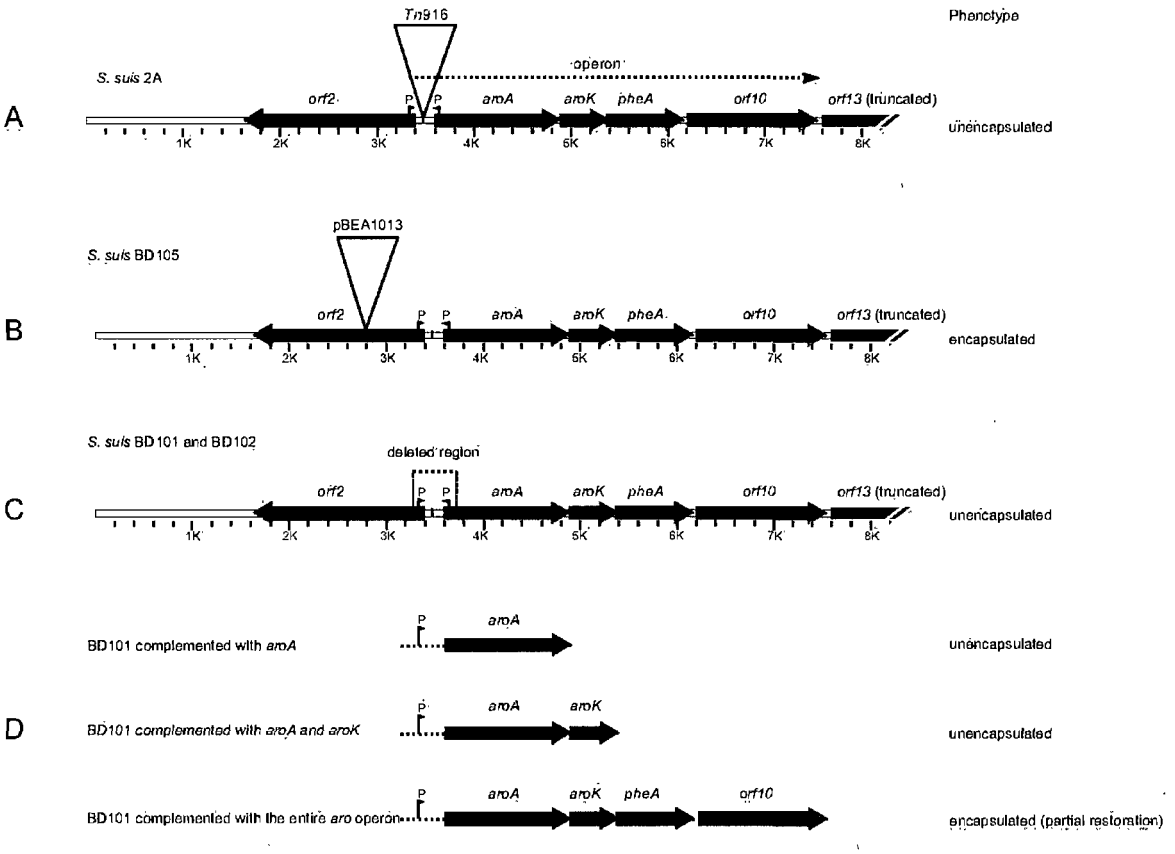

Figure 35. Article IV, Figure 1. Genetic organization of the *S. suis aro* region and strategies followed for mutant generation.

A) Schematic representation of a 8.4 kb genomic DNA surrounding the insertion site of transposon Tn*916* in mutant 2A. *In silico* analysis and Northern blot experiments showed the presence of 6 ORFs, 4 of which are organised in an operon (*aro* operon). The putative promoters for the *orf2* and the *aro* operon in *S. suis* virulent strain S735 are indicated. B) The *orf2* was inactivated in mutant strain BD105 (*orf2*::pBEA1013) by insertion of a recombinant plasmid. Inactivation of the *orf2* does not affect capsule production. C) Deletion of a region comprising the putative *aro* promoter in both reference strain S735 and field strain 31533 results in the abolishment of the encapsulated phenotype. D) Complementation of mutant strain BD101with different constructions under the control of the cognate *aro* promoter. Capsule production was restored partially only when the construction comprised the entire *aro* operon.

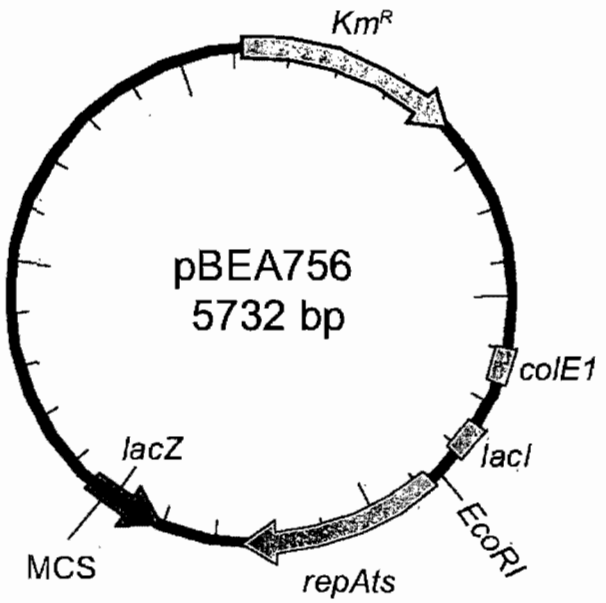

Figure 36. Article IV, Figure 2. Plasmid pBEA756 is a thermosensitive suicide vector derived from plasmid pCIV2 [23].

It carries a Km resistance element, an *E. coli colE1* and a thermosensitive Gram positive replication origin *repAts*. MCS, multiple cloning sites.

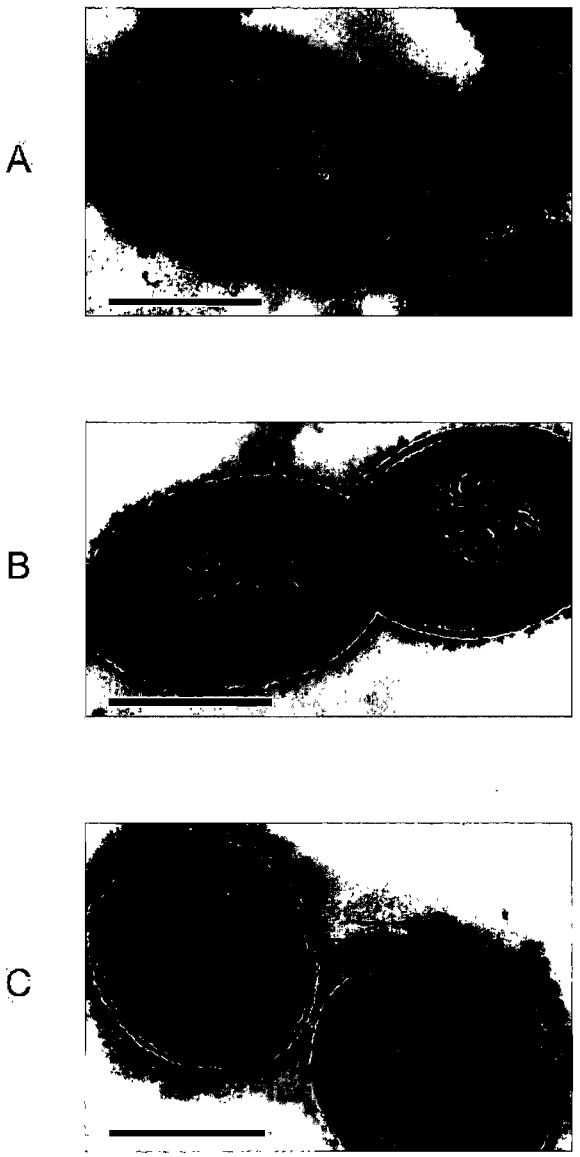

Figure 37. Article IV, Figure 3. Transmission electron microphotographs of thin sections of *S. suis* WT and mutants used in this study.

A) Serotype 2 reference strain S735, which shows a thick capsule characteristic of this serotype. Original magnification 37,500 X; B) Mutant strain S735 Δ*aro*, which exhibits an unencapsulated phenotype; C) Complemented strain CA119 (strain BD 101complemented with a recombinant plasmid carrying the entire *aro* operon under the control of its cognate promoter), which shows a partially restored capsular phenotype. Original magnification for B and C: 62,500 X. Bar = 0.5 µm.

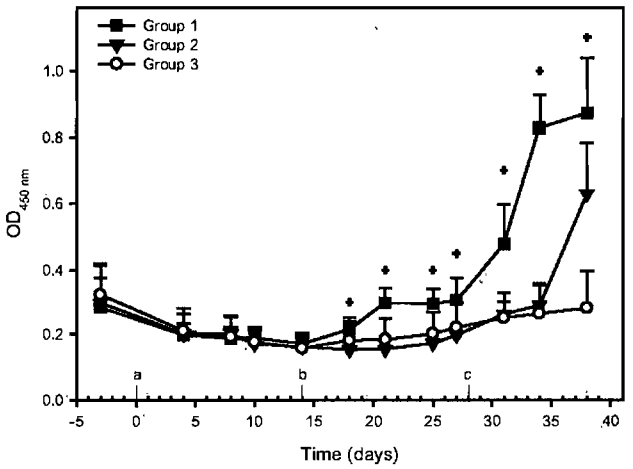

Figure 38. Article IV, Figure 4. Antibody response in the 3 groups of animals used in this study. Animals in group 1 (closed squares) received two doses of *S. suis* mutant strain BD101 intramuscularly at days 0 (a) and 14 (b), respectively. Animals in group 2 (closed triangles) and 3 (open circles) were not vaccinated. Animals in group 3 were not challenged (control group) while those in groups 1 and 2 were challenged with *S. suis* serotype 2 virulent strain S735 at day 28 (c). + Significant differences between vaccinated and non-vaccinated groups 1 and 2 (t-test; $P<0.05$).

ARTICLE V

"Serotype distribution and production of muramidase-released protein, extracellular factor and suilysin by field strains of *Streptococcus suis* isolated in the United States."

Nahuel Fittipaldi and Marcelo Gottschalk.

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Author contributions: NF and MG designed research; NF performed research, NF and MG analyzed data; NF wrote the manuscript and MG revised the manuscript.

Although this article is part of, and describes research carried out during, the graduate studies of the candidate, the subject was not part of the original research plan for the candidate. Therefore, it is included in the Appendix section.

Details on the role of the candidate in the conception of the article: I contributed to research design, performed research, analyzed data and wrote the paper.

Serotype distribution and production of muramidase-released protein, extracellular factor and suilysin by field strains of *Streptococcus suis* isolated in the United States

Nahuel Fittipaldi and Marcelo Gottschalk*.

Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Qc J2S 7C6, Canada

Running title: Virulence markers in *Streptococcus suis* U.S. isolates.

Keywords: *Streptococcus suis*, virulence markers, MRP, EF, Suilysin, swine

* Corresponding author. Tel.: +1 450 773-8521 ext 18389; fax +1 450 778-8108. E-mail address: [redacted]

Abstract

Streptococcus suis is an important swine pathogen and a zoonotic agent. Differences in virulence have been noted between the 35 described serotypes, the serotype 2 being considered the most virulent. In this study, we evaluated the serotype distribution among 100 isolates recovered from diseased pigs in the United States. Results showed that serotype 3 (20 % of the isolates) and serotype 2 (17%) were the most prevalent. We also evaluated the presence in these isolates of the genes *sly*, *epf* and *mrp*, coding for the virulence-associated markers suilysin (SLY), extracellular factor (EF) and muramidase-released (MRP) protein, respectively. The effective production of the markers by the strains was also verified. Questioning the usefulness of PCR for the assessment of the production of these markers in *S. suis*, results showed that presence of gene did not always correlate with actual expression of the respective protein. In the case of MRP, this was due, in most cases, to frameshift mutations at the 5' end of the gene resulting in premature stop codons. The most prevalent phenotypes among U.S. strains were $MRP^{+}EF^{-}SLY^{-}$ (40 %) and $MRP^{-}EF^{-}SLY^{+}$ (35 %). Serotype distribution as well as production of virulence markers greatly differed from those reported in several European countries, particularly for serotype 2. On the other hand, our results for the U.S. *S. suis* isolates are similar to those reported for Canadian strains, suggesting a common status in North America.

Introduction

Streptococcus suis is an important agent of meningitis, septicemia, arthritis, pneumonia, and endocarditis in pigs and is also a zoonotic agent (Gottschalk et al., 2007; Higgins and Gottschalk, 2006). Despite increasing research in recent years, which has in part been boosted by the 2005 deadly human outbreak in China (Yu et al., 2006), the pathogenesis of *S. suis* infections remains poorly known. Differences in virulence among the 35 described serotypes is well documented (Higgins and Gottschalk, 2006). *S. suis* serotype 2 is usually reported as the serotype most frequently associated with disease in both swine and humans, although other serotypes may also be involved in invasive diseases (Higgins and Gottschalk, 2006). In addition, important differences in virulence also exist among strains of the same serotype, particularly those belonging to serotype 2 (Berthelot-Herault et al., 2005; Quessy et al., 1995; Vecht et al., 1992). The capsule, a fibronectin/fibrinogen binding protein and a serum opacity-like factor have been reported to be involved in the virulence of this pathogen (Baums et al., 2006; Chabot-Roy et al., 2006; de Greeff et al., 2002; Smith et al., 1999). More recently, modifications of the cell wall such as the D-alanylation of the lipoteichoic acids and the N-deacetylation of the peptidoglycan have been shown to contribute to the virulence traits of this pathogen (Fittipaldi et al., 2008a; Fittipaldi et al., 2008b). In addition, a thiol-activated hemolysin known as suilysin (SLY) may play a role in *S. suis* virulence (Jacobs et al., 1994). Encoded by the *sly* gene, SLY has a cytotoxic effect, is able to affect complement resulting in impaired opsonophagocytosis and might allow penetration of *S. suis* into deeper tissues (Chabot-Roy et al., 2006; Lalonde et al., 2000; Vanier et al., 2004). However, SLY is not essential for virulence, as demonstrated by the fact that isogenic *sly* mutants did not show any impairment in their ability to induce disease when compared to the parent SLY^+ strain (Lun et al., 2003).

Expression of SLY, together with those of the LPXTG-protein known as muramidase-released protein (MRP, 136 kDa) and the secreted protein extracellular factor (EF, 110 kDa), have been used as phenotypic markers to distinguish virulent from avirulent isolates of *S. suis* serotype 2, (Higgins and Gottschalk, 2006). These MRP and EF proteins, and their molecular weight variants designated by an asterisk (MRP*, EF*), are encoded by the genes *mrp* (Smith et al., 1992) and *epf* (Smith et al., 1993), respectively. As for SLY, the role of MRP and EF proteins

has not been clearly defined but they do not appear to confer virulence directly, as demonstrated with the use of gene knockout technology (Smith et al., 1996). However, there is a positive association between the presence of these proteins and virulence in European and Asian strains of *S. suis* (Gottschalk et al., 2007). Indeed, in several countries of these continents, $MRP^{+}EF^{+}SLY^{+}$ strains of the serotype 2 are isolated mainly from diseased pigs and are associated with severe clinical signs of disease after experimental infection. In contrast, $MRP^{-}EF^{-}SLY^{-}$ strains have been frequently isolated from healthy pigs and have been reported to be non-pathogenic after experimental inoculation (Allgaier et al., 2001; Vecht et al., 1992). On the other hand, the $MRP^{+}EF^{*}$ serotype 2 strains, which produce high molecular weight variants of EF, have been reported to be less virulent for young pigs (Vecht et al., 1992). In addition, to the best of our knowledge, avirulent strains possessing MRP, EF and suilysin have not been reported. However, some European and most Canadian virulent isolates do not produce any of these 3 factors (Berthelot-Herault et al., 2000; Gottschalk et al., 1998; Quessy et al., 1994; Segers et al., 1998).

Despite the fact that many studies addressing the prevalence of MRP, EF and SLY have been carried out with European and Canadian isolates, the prevalence of MRP, EF and SLY in the United States (U.S.), by far the largest North American swine producer, remains poorly known. Indeed, only one study has conducted a phenotypic evaluation of the presence of MRP and EF in selected serotype 2 isolates (Galina et al., 1996). In the present study, we carried out a genotypic and phenotypic assessment of the presence of these virulence markers in *S. suis* isolates from the U.S.

Materials and methods

S. suis isolates. A total of 100 *S. suis* field strains isolated in the U.S. between January, 2003 and December, 2005 were used in this study. The isolates had been submitted as part of routine diagnostic procedures to the University of Minnesota Veterinary Diagnostic Laboratory or the Iowa State University Veterinary Diagnostic Laboratory and identified as *S. suis* using standard biochemical tests. Criteria for inclusion in this study were as follows: a) isolates were from different farms b) pigs must have shown clinical signs consistent with *S. suis* infection; c) isolates must have been obtained from organs at necropsy; (i.e. tonsillar or nasal swabs were not accepted) and d) isolates obtained in conjunction with other primary bacterial pathogens

(i.e. *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*) were not accepted. Isolates originated from 17 states (Table S1). The majority of the isolates were from the major swine-producing states Iowa (28 isolates) and North Carolina (25 isolates). Forty-four samples were from cases of septicemia, isolated from the liver or spleen (both organs in most cases). Fourteen samples were isolated from the central nervous system (either from brain, meninges or cerebrospinal fluid). Forty isolates were from cases of pneumonia (39 from lung and 1 from pleura). Finally one strain was isolated from the heart and one from joints. Serotyping was performed by a coagglutination test using rabbit hyperimmune sera and a reference panel of all 35 serotypes of *S. suis,* as previously described (Gottschalk et al., 1993; Higgins and Gottschalk, 1990). *S. suis* serotype 2 strains S735 ($MRP^+EF^*SLY^+$), P 1/7 ($MRP^+EF^+SLY^+$), D282 ($MRP^+EF^+SLY^+$), T15 ($MRP^-EF^-SLY^-$) and 89-1591 ($MRP^-EF^-SLY^-$), originating from our collection, were used as controls.

Strain culture and genomic DNA preparation. *S. suis* strains were grown in Todd-Hewitt broth (Becton Dickinson, Sparks, MD, USA) at 37°C for 16 h. Genomic DNA was prepared from 10 ml of overnight (ON) cultures using the QIAamp DNA Minikit (Qiagen Inc., Valencia, CA, USA) or the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) as recommended by the respective manufacturers.

PCR assay for sly and orf102. Amplification of the *sly* gene was achieved by using previously described (King et al., 2001) conditions and primers SLY3 and SLY2 (all oligonucleotides primers were from Invitrogen, Burlington, ON, Canada and are presented in Table 1). The *orf102* gene, which has been described to be mutually exclusive with the *sly* gene (King et al., 2001; Takamatsu et al., 2002) was amplified using primers TEF-1025 and TEF-1026. The concentrations of PCR reagents per reaction (final volume 25 µl) were as follows: 1X XL Buffer II (Invitrogen), 0.8 mM each dNTP, 1.5 mM $Mg(OAc)_2$, 0.5 µM of each primer, 1 U r*Tth* DNA Polymerase (Invitrogen) and 1 µl of genomic DNA as template. PCR parameters consisted of 35 cycles of 94°C for 20 sec, 55°C for 30 sec, 72°C for 1 min with an initial denaturation step at 94°C for 2 min and a final extension step at 72°C extension for 7 min. PCR amplicons (1492 bp for *sly* and 481 bp for *orf102*) were electrophoresed on 1 % agarose gels and visualized by UV transillumination after ethidium bromide staining.

PCR assay for epf. Amplification of the *epf* gene was achieved by using the previously described conditions and primers epf-F and epf-R (Table 1) (Wisselink et al., 1999). This PCR test was able to discriminate between 6 variants of the *epf* gene (amplicons of 626, 1278, 1505, 2313, 2537 and 2993 bp, respectively) (Wisselink et al., 1999).

Sequencing of the mrp gene. Full length *mrp* was amplified from most isolates using one or more of different primer pairs (one of TEF-1067, TEF-1068, TEF-1069, TEF-1070, TEF-1071, TEF-1072, TEF-1073, or TEF-1074 with one of TEF-1075, TEF-1076, TEF-1077, TEF-1078, TEF-1079, TEF-1080, or TEF-1081). Primer sequences are listed in Table 1 and their annealing positions to the *mrp* genetic region are depicted in Fig. 1A. The concentrations of PCR reagents per reaction (final volume 25 µl) were those described above for *orf102*. Amplifications were as follows: 94°C for 2 minutes, 35 cycles of 94°C for 20 seconds, 55°C for 30 seconds and 72°C for 4 minutes, 72°C for 10 minutes. PCR amplicons ranging from 4 to 5 kb as determined by agarose gel electrophoresis were considered positive, purified and sequenced as described below. When the full length *mrp* gene could not be amplified, a variable size fragment was amplified using the same conditions but with primers TEF-1033 and TEF-1042 (Table 1). PCR-generated *mrp* amplicons were purified using either the QIAquick PCR Purification Kit (Qiagen) or the MinElute 96UF kit (Qiagen) according to the manufacturer's recommendations. Sequencing reactions were performed in a final volume of 13 µl containing 5 µl of purified amplicon as template, 2 µl BigDye Terminator v. 3.0 Chemistry kit, 1.5 µl ABI 5X Sequencing Buffer (Applied Biosystems, Foster City, CA, USA), and 0.23 µM primer. Primer walking was used for the complete and overlapping coverage of the *mrp* gene. Primers were TEF-1075, TEF-1033, TEF-1034, TEF-1051, TEF-1052, TEF-1053, TEF-1054, TEF-1040, TEF-1057, TEF-1082, TEF-1042, TEF-1083, TEF-1084, TEF-1046, and TEF-1067 (Table 1 and Fig. 1A). Reactions consisted of 50 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Products were then purified using either the Performa DTR 96-well standard plate kit (Edge BioSystems, Gaithersburg, MD, U.S.A.) or CentriSep Spin Columns (Princeton Separations, Adelphia, NJ, U.S.A.) according to manufacturer's instructions. Samples were analyzed on an ABI 3730 DNA Sequencer (Applied Biosystems).

Hemolytic assay for suilysin. Citrated horse red blood cells (hrbc, 25 ml) were washed with 25 ml of Dulbecco's Phosphate Buffered Saline (PBS), pH 7.3, (Gibco, Grand Island, NY, USA) and centrifuged at room temperature (RT) for 10 min at 1000 rpm in a Sorvall RT6000 centrifuge with a Sorvall H1000B rotor. The supernatant was removed and the pellet washed two more times as described above. The resulting pellet of washed citrated hrbc was brought up to a final volume of 100 ml in PBS. The hemolytic activity of *S. suis* isolates was assessed by gently mixing 25 µl of ON bacterial culture supernatants with 25 µl of washed citrated hrbc in 96-well Costar 3799 plates (Corning Incorporated, Corning, NY, USA). Plates were incubated at 37°C with 5% CO_2 for 1 h followed by incubation at room temperature for 16-24 h. Results were evaluated visually. Wells in which hrbc were completely lysed were considered positive.

Western-blot assays for MRP and EF. Supernatants obtained after centrifugation of ON *S. suis* cultures in THB were concentrated 10-fold by Ultrafree-MC centrifugal filter devices (Millipore Corp., Bedford MA, USA). Samples were prepared for the E-PAGE protein electrophoresis system (Invitrogen) and 10 µl of samples were run on 8% E-PAGE 48 gels as per manufacturer's instructions. After electrophoresis, proteins were transferred to nitrocellulose filters (BioRad, Hercules, CA, USA). The blots were blocked with 2 % casein and incubated with a 1:200 dilution of monoclonal antibodies against either MRP or EF (Vecht et al., 1992). After extensive washes with blocking solution, the membranes were incubated with peroxidase-conjugated, affinity purified rabbit anti-mouse antibodies (Rockland, Gilbertsville, PA, USA) diluted 1:10000. The membranes were washed in Tris-Buffered Saline, pH 7.6, prior to detection with TMB 1-Component Membrane Peroxidase Substrate (KPL, Gaithersburg, MD, USA).

Results and discussion

Serotyping. We first determined the serotype of the isolates included in the panel. Results showed that of the 100 isolates, serotype 3 (N=20) was the most prevalent, followed by serotypes 2 (N=17), 7 (N=13), 8 (N=11), 6 (N=9) and 1/2 (N=8). These 6 serotypes together accounted for 78 % of the isolates. Four isolates belonged to serotype 5. Serotypes 9, 1 and 23 comprised three isolates each; serotype 18 two isolates, and serotypes 10, 12, 14, and 30, one isolate each. Finally, three strains were nontypable. These strains were confirmed as belonging to the *S. suis* species by using a previously described specific PCR test targeting the *gdh* gene

(Okwumabua et al., 2003) (data not shown). Serotype determination remains a valuable tool used by veterinary practitioners and diagnosticians to understand the epidemiology of a particular outbreak and/or to increase the possibility of success of a vaccination program within a herd. To the best of our knowledge, this is the first report of serotype distribution for country-wide obtained *S. suis* U.S. isolates. Interestingly, in the present study, the predominant *S. suis* serotypes as well as their prevalence were comparable to those reported for Canadian isolates (Messier et al., 2008), which seems to confirm a previous hypothesis suggesting that the prevalence of *S. suis* serotypes are similar in these two North American countries (Higgins and Gottschalk, 2006). This was expected since many Canadian piglets are routinely sent to growing/finisher units in the U.S. Further studies with a larger number of U.S. isolates would be needed to definitively sustain this hypothesis. Previous reports analyzing *S. suis* isolates in the state of Minnesota have shown that serotype 2 was the most prevalent in that state among field strains obtained during years 1989 and 1990 (27 to 34%) and years 1992 and 1993 (with a surprising 77 %) (Galina et al., 1992; Galina et al., 1996). In contrast, data obtained in this study showed that only 17 % of isolates of U.S. origin belonged to serotype 2, a percentage that is similar to that reported in Canada and considerably lower than those reported in European countries for this serotype (Berthelot-Herault et al., 2000; Messier et al., 2008; Wisselink et al., 2000).

SLY and EF. The presence of the *sly* gene was determined by a PCR assay targeting the *sly* gene (King et al., 2001). Forty-nine isolates were positive for an amplicon of the expected size (Table 2). The *sly* gene lies between the *orf101* gene, encoding for a putative haloacid dehalogenase hydrolase and the *nanE* gene, which encodes for a putative *N*-acetylmannosamine-6-phosphate epimerase (King et al., 2001; Takamatsu et al., 2002). In strains lacking *sly* an alternative genetic structure has been described, with *orf102* (a gene of unknown function) occupying the place of *sly* (King et al., 2001; Takamatsu et al., 2002). In order to validate our *sly* PCR results we designed PCR primers to amplify an *orf102* fragment. There was a strong agreement between the two assays: 95 % of the isolates contained either the *sly* or the *orf102* genes. However, 5 isolates were negative for both *sly* and *orf102* PCR assays. Although we cannot exclude that absence of amplification in these 5 isolates may reflect mutations affecting annealing of the primers used, our results may also result from a novel

organization of the region without any of the *sly* or *orf102* genes. Further studies are needed to evaluate this hypothesis. Phenotypically, the 51 isolates that were negative for the *sly* gene by PCR were also negative for hemolytic activity. On the other hand, of the 49 isolates that were determined to be positive for *sly* by PCR, only 39 were able to lyse citrated hrbc (Table 2). The remaining 10 isolates did not show hemolytic activity. Although we have not performed sequencing of the amplicons obtained by PCR for these 10 isolates, we hypothesize that the *sly* alleles in these isolates are likely to present mutations resulting in the absence of expression of a functional protein. Therefore, for any given *S. suis* isolate, PCR results for *sly* should be interpreted with caution and should not replace the effective evaluation of the hemolytic capacity of the strain.

We also evaluated the presence of the *epf* gene by using a previously described PCR test (Wisselink et al., 1999). Thirty-one isolates were positive for the *epf* gene (Table 2). Of them, 6 presented the expected size for the originally described *epf* gene, while from the remaining samples amplicons had sizes compatible with the *epf* PCR variant epf*v3 (Wisselink et al., 1999) (data not shown). However, only two isolates expressed EF, as determined by Western blotting with an anti-EF monoclonal antibody (Vecht et al., 1992). As for the *sly* gene, we did not perform sequencing of the *epf* amplicons generated by PCR. Therefore, we cannot exclude the presence of mutations affecting the actual expression of EF in these *epf* PCR positive strains. However, the only two Western-blot EF positive strains belonged to serotype 2. These strains were also the only serotype 2 strains positive for *epf* by PCR. Indeed, most of the *epf* PCR positive strains belonged to serotypes other than 2 (Table 2). Since the monoclonal antibodies against EF were generated using serotype 2 EF protein (Vecht et al., 1992), it might also be hypothesized that this monoclonal antibody recognizes an EF epitope present in serotype 2 strains only.

MRP. When we analyzed the expression of MRP by Western-blot using specific monoclonal antibodies, 46 isolates were found to be positive. Of them, 43 isolates presented MRP-reactive bands ranging in size from 120 to 150 kDa, molecular weights (MW) that are consistent with previous reports (Silva et al., 2006; Smith et al., 1992). On the other hand, 3 isolates were positive for a much lower MW band (76 kDa) (Table 3). Using different combinations of external PCR primers designed to amplify the entire MRP open reading frame

(see materials and methods and Fig. 1A), amplicons ranging in size from 4 kb to 5 kb (compatible with the full length *mrp* gene and the reported *mrp* variants) were obtained for 82 isolates (Table 3). However, we failed to observe amplification for 18 isolates. We therefore designed primers annealing within the *mrp* coding sequence and obtained amplicons for 10 of these isolates. The remaining 8 isolates were also negative with these internal primers (Table 3).

To investigate the discrepancy between the number of MRP positive isolates by Western-blot and the number of isolates containing putative full-length *mrp* gene copies, the PCR amplicons were sequenced. Sequence data have been submitted to GenBank and are available under Accession Numbers FJ685523 to FJ685613. Sequencing results divided the 92 isolates in 3 defined groups. A first group of 43 isolates, which matched perfectly the isolates positive for MRP by Western blot, presented an intact *mrp* open reading frame with the reported initiator ATG codon (Smith et al., 1992). A second group of three isolates presented nonsense mutations at about 2.1 kb from the ATG initiator codon. In silico translation predicted LPXTG-less, C-terminally truncated polypeptides of 707 amino acids for these three isolates. In strong agreement, these 3 isolates were those that showed a truncated 76 kDa band by Western blot (data not shown). Finally, for 46 isolates, sequencing revealed the presence of frameshift mutations at different short distances from the reported initiator ATG codon, which resulted in premature stop codons (data not shown). Consistently, all the isolates possessing such a defective 5' end *mrp* gene copy had tested negative for MRP by Western blot. Of note, except for the frameshift mutations, all these Western-blot negative isolates presented a "full length" *mrp* gene. Interestingly, in a recent study, a PCR test using primers which anneal to the 3' end of the *mrp* gene was described which allows for the identification of *mrp* gene variants (Silva et al., 2006). That study reported a very good agreement between PCR positive results and expression of MRP in a panel of European strains. However, given the high percentage of frameshift mutations in the *mrp* gene found in the present study resulting in absence of expression of MRP, at least for North American strains, it seems likely that the *mrp* variant PCR is of limited use for the assessment of MRP production.

Irrespective of the actual expression of MRP, sequencing also revealed the presence of 3 distinct *mrp* genotypes among the 92 sequenced U.S. isolates. The first of these genotypes,

referred to in this study as European (EU), is represented by the *mrp* sequence originally reported for *S. suis* European strains D282 (GenBank Accession Number X64450) (Smith et al., 1992) or P 1/7 (gene SSU_0706, available online at the Sanger Institute, http://www.sanger.ac.uk/Projects/S_suis/). The second genotype, referred to here as North American (NA) 1, is represented by the Canadian isolate 89/1591 (available online at http://genome.jgi-psf.org/draft_microbes/strsu/strsu.home.html), even though this strain does not actually express MRP (Quessy et al., 1995). A third, previously unreported genotype, referred to as NA2, was identified in this study in 14 of the tested isolates. Strain PAH-146 (GenBank Accession Number FJ685609) represents this genotype. The three genotypes are very similar at the 5' and 3' ends while variation is observed in the central part of the gene. Indeed, predicted translated sequences of these 3 genotypes showed 99 % identity for the first 220 amino acids and the C-terminal last 147 amino acids. On the other hand, between 50 to 60% identity for the more central adjoining 475 amino acids was observed between the genotypes (Fig. 1B). Distribution-wise, and irrespective of whether MRP was expressed or not, 74 isolates belonged to the NA1 genotype, 14 were NA2, and 4 were of the EU type.

Finally, sequencing data revealed that the heterogeneities in MW observed by Western-blot were 100% consistent with the open reading frames identified by sequencing and were mainly accounted for by the different occurrences (0 to 3) of 137 amino acid repeats located within the C-terminal half of the protein (Fig. 1B). Most isolates presented one such repeat. Therefore, the overall genotypic structure of the *mrp* locus can be characterized by a combination of 1) a 5' end without (intact, I) or with (defective, D) frameshift mutations, 2) one of the 3 related genotypes (EU, NA1, NA2), and 3) the number of repeats (0 to 3). The most prevalent *mrp* genotype among the U.S. *S. suis* isolates was a combination of an intact 5' end of the ORF, the NA1 variable region, and one repeat ("I/NA1/1"), representing over 25 % of the isolates. The next most prevalent *mrp* genotype was D/NA1/1. These two *mrp* genotypes accounted for approximately half of the isolates in the study.

Phenotypes. Studies carried out with European strains, especially those of serotype 2 have shown that the MRP and EF proteins as well as SLY are strongly associated with the virulence of ***S. suis*** (Jacobs et al., 1995; Wisselink et al., 2000). On the other hand, these proteins

are not frequently associated with Canadian virulent strains (Gottschalk et al., 1998). Results of the present study show that, taking all the serotypes together, the two most prevalent phenotypes found for U.S. isolates are $MRP^+EF^-SLY^-$, which accounted for 40 % of the isolates and $MRP^-EF^-SLY^+$ (35 % of the isolates) (Table 3). The triple negative phenotype $MRP^-EF^-SLY^-$ accounted for 16 % of the isolates. EF* expressing strains were not found in our U.S. panel, which is in accordance with the situation in Canada (Higgins and Gottschalk, 2006), although, as discussed above, some epf*v3 positive results were obtained by PCR for serotypes other than serotype 2. Interestingly, among isolates belonging to serotype 2, only 3 different phenotypes were observed. The most prevalent phenotype among serotype 2 strains was $MRP^+EF^-SLY^-$ (76%), followed by the triple negative $MRP^-EF^-SLY^-$ phenotype (12%). Although the observed prevalence of MRP among serotype 2 isolates was higher than that reported in Canada, the prevalence of EF and SLY among U.S. strains of serotype 2 was in agreement with that reported for Canadian strains. Indeed, the triple positive phenotype $MRP^+EF^+SLY^+$ was observed for only two serotype 2 strains. Interestingly, although most MRP positive serotype 2 strains presented a NA1 *mrp* genotype, these 2 $MRP^+EF^+SLY^+$ isolates showed an EU *mrp* genotype. These results suggest that these 2 isolates might be of European origin and might have been introduced to the United States through the importation of animals. Further phylogenetic studies are needed to evaluate this proposal.

Our results suggest a common situation regarding the distribution of different serotypes of *S. suis* in the United States and Canada. The production of virulence markers such as MRP, EF and SLY by U.S. isolates also differs from that reported for European strains but it is similar to that reported in Canada. Interestingly, a higher association of *S. suis* cases with the immunosuppressive infection caused by the porcine reproductive and respiratory syndrome virus has been reported in North America while, on the other hand, in Europe, *S. suis* is most often the primary agent of infection (Higgins and Gottschalk, 2006). It has been hypothesized that European virulent serotype 2 strains, especially those with the phenotype $MRP^+EF^+SLY^+$, may be more virulent than North American strains (Gottschalk and Segura, 2000) and, indeed, greater virulence was demonstrated for a serotype 2 European $MRP^+EF^+SLY^+$ strain compared to a virulent Canadian $MRP^-EF^-SLY^-$ strain using a standardized experimental infection method (Berthelot-Herault et al., 2005). In addition, human *S. suis* infections are much less common in

North America than in Europe and Asia and they have thus far not resulted in mortality of the patients, whereas death has been more common among European and Asian *S. suis* human cases (Gottschalk et al., 2007). Results presented here further suggest that North American and European strains are dissimilar. However, differences in virulence among strains from these two origins may probably not be explained solely based on the differences in MRP, EF, and SLY production. The availability of genomic data from the ongoing genome sequencing of European (P 1/7) and North American (89-1591) strains will hopefully help to resolve this dichotomy.

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Article V Tables

Table XIX. Article V, Table 1. Oligonucleotide primers used in this study.

Primer name	Sequence (5'-3")	Use (Reference)
JP4	GCAGCGTATTCTGTCAAACG	Species detection (Okwumabua et al., 2003)
JP5	CCATGGACAGATAAAGATGG	Species detection (Okwumabua et al., 2003)
Sly-2	ACTCTATCACCTCATCCGCv	*sly* amplification (King et al., 2001)
Sly-3	ACTCTATCACCTCATCCGC	*sly* amplification (King et al., 2001)
TEF-1025	ACGAGAAAACCTTGCGACTG	PCR for *orf102* amplification
TEF-1026	CTGGATTGATAGGAGTGTTTG	PCR for *orf102* amplification
TEF-1033	CGAAACAACAATTTTCGATTCGTAAG	*mrp* sequencing
TEF-1034	GGAGCAATTGCTGCACCATTTGG	*mrp* sequencing
TEF-1040	CAGATGTGGACCGTAGACCAG	*mrp* sequencing
TEF-1041	CAGCTAAGACAACCGGTACAG	*mrp* sequencing
TEF-1042	GTTGGTGGTACATAACCCTTACCTGG	*mrp* sequencing
TEF-1046	CCAACAACACCTGAGAAGGAAACTCC	*mrp* sequencing
TEF-1051	CCAAATGGTGCAGCAATTGCTCC	*mrp* sequencing
TEF-1052	CTGTCCTGCAATAGTTTGAAGAC	*mrp* sequencing
TEF-1053	GTCTTCAAACTATTGCAGGACAG	*mrp* sequencing
TEF-1054	GACGCGATATTTGCTACCTTG	*mrp* sequencing
TEF-1057	CCAGCTTCAATCTTACCTGTTGTTGG	*mrp* sequencing
TEF-1067	GCGAACTCTAAGATGTAACTCTGGAC	*mrp* sequencing
TEF-1068	GAGTAGTGACTGTTGGAGGTTGG	*mrp* sequencing
TEF-1069	CCCCACACAGCTCCAACAGTC	*mrp* sequencing
TEF-1070	GCGTCAGAGGATTGCGAATATCTG	*mrp* sequencing
TEF-1071	CTCAAAGGATAGAGAGTTTGGAGC	*mrp* sequencing
TEF-1072	CAGGTATTCCAAGACCTTGCGTTC	*mrp* sequencing
TEF-1073	GCGGTTGACCTGCTTCTTTCCTATC	*mrp* sequencing
TEF-1074	AATCATTGCCAGACCAACATCATC	*mrp* sequencing
TEF-1075	TTTCAAGGCTTTGATACAAAGAGTAG	*mrp* sequencing
TEF-1076	GTATAATACCTCGRATGATTGCGGGAG	*mrp* sequencing
TEF-1077	GCATTTAAAGTTATTTGTAGTATAATACCTCG	*mrp* sequencing
TEF-1078	ATGGAGCTAATATTTTTGCGGTGTCG	*mrp* sequencing
TEF-1079	TGAGCGTTTGGCAAGAGAGC	*mrp* sequencing
TEF-1080	CAATCGCAAACTCCGTTTGG	*mrp* sequencing
TEF-1081	GCCGATGTTTGGAATGCTG	*mrp* sequencing
TEF-1082	CAACAGAGCCAGACGAGCCAATC	*mrp* sequencing
TEF-1083	GTTGCCATCTTCGTCAGTAACAG	*mrp* sequencing
TEF-1084	CATCAACTACAGTACGCACATACTC	*mrp* sequencing

Table XX. Article V, Table 2. Prevalence of suilysin and EF among the 100 U.S. *Streptococcus suis* isolates.

Serotype	N	Suilysin			EF	
		sly (PCR)	*orf 102* (PCR)	Hemolysis	PCR	Western-blot
1	3	0	2	0	0	0
2	17	2	14	2	2	2
3	20	5	14	5	5	0
4	9	9	0	9	9	0
5	4	3	1	3	4	0
7	13	10	3	3	3	0
8	11	10	1	8	1	0
9	3	1	2	1	1	0
10	1	1	0	1	1	0
12	1	1	0	1	0	0
14	1	1	0	1	0	0
18	2	1	1	1	1	0
23	3	3	0	3	3	0
30	1	1	0	1	0	0
1/2	8	0	8	0	1	0
NT [a]	3	1	0	0	0	0
Total	100	49	46	39	31	2

[a] Non typable

Table XXI. Article V, Table 3. Prevalence of MRP among the 100 U.S. *Streptococcus suis* isolates

Serotype	N	*mrp* gene	Intact N-terminus	Western-blot
1	3	3	3	3
2	17	17	15	15
3	20	20	13	13
4	9	9	0	0
5	4	3	0	0
7	13	10	0 [b]	0 [b]
8	11	11	1	1
9	3	1	0	0
10	1	1	0	0
12	1	1	1	1
14	1	1	0	0
18	2	2	1	1
23	3	3	0	0
30	1	1	0	0
1/2	8	8	8	8
NT [a]	3	1	1	1
Total	100	92	43	43

[a] NT non typable

[b] Three isolates presented a truncated *mrp* gene at 2.1 kb from the reported initiator ATG codon (Smith et al., 1992). A corresponding truncated MRP (approx 76 KDa) was detected by Western blot.

Table XXII. Article V, Table 4. Distribution of MRP, EF and SLY phenotypes among the 100 U.S. *Streptococcus suis* isolates.

Serotype	N	Phenotype				
		$MRP^+EF^-SLY^-$	$MRP^-EF^-SLY^+$	$MRP^-EF^-SLY^-$	$MRP^+EF^-SLY^+$	$MRP^+EF^+SLY^+$
1	3	3	0	0	0	0
2	17	13	0	2	0	2
3	20	13	5	0	0	0
4	9	0	9	0	0	0
5	4	0	3	1	0	0
7	13	0	3	7	0	0
8	11	1	6	2	1	0
9	3	0	1	2	0	0
10	1	0	1	0	0	0
12	1	0	0	0	1	0
14	1	0	1	0	0	0
18	2	1	1	0	0	0
23	3	0	3	0	0	0
30	1	0	1	0	0	0
1/2	8	8	0	0	0	0
NT [a]	3	1	0	2	0	0
Total	100	40	35	16	2	2

[a] NT non typable

Table XXIII. Article V, Supplemental Table 1. State of origin of the 100 U.S. isolates used in this study.

State	Number of isolates
Iowa	29
North Carolina	25
Oklahoma	7
Nebraska	7
Missouri	6
Minnesota	5
Indiana	5
Illinois	3
Kentucky	2
Wisconsin	2
Utah	2
Texas	2
Virginia	1
Kansas	1
Ohio	1
Michigan	1
Colorado	1
Total	100

Article V Figures

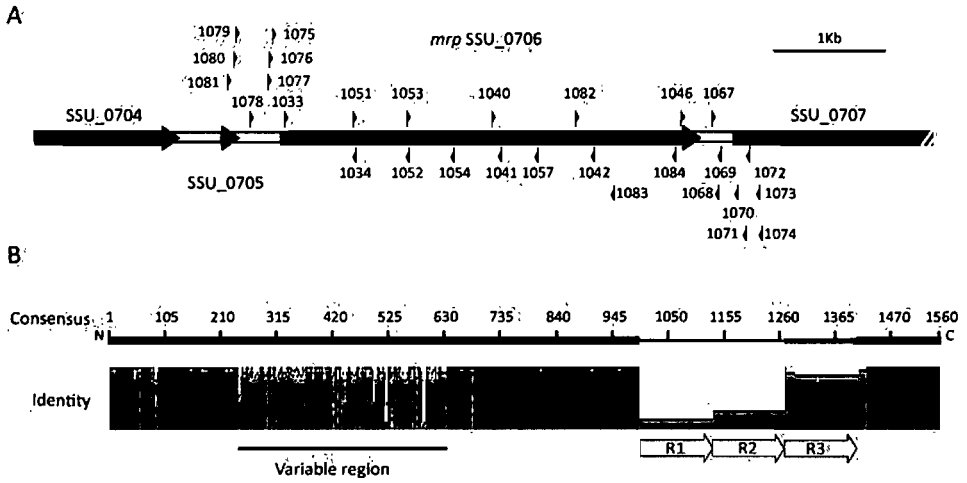

Figure 39. Article V, Figure 1. The *mrp* genetic region and positions of primers used in this study.

A) Schematic representation of the *mrp* genetic region in serotype 2 strain P1/7 (available at http://www.sanger.ac.uk/Projects/S_suis/). Arrows indicate the annealing position of the primers used for the determination of the presence and sequencing of the *mrp* gene. B) Sequence alignment of translated predicted amino acid sequences of MRP for the isolates used in this study. Sequences were aligned using the CLUSTALW software available at http://www.ebi.ac.uk/Tools/clustalw/. The N-terminal and C-terminal regions presented 99% identity among the isolates. On the other hand, only between 50 to 60 % identity was observed for the central region among isolates, which could be clustered on three different genotypes (EU, NA1 and NA2) based on their sequence for this variable region. Finally, strains were shown to possess a variable number (0 to 3) of a 137 aa repeat (indicated by the arrows).