Review
Future Microbiology

Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent *Streptococcus suis*

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Streptococcus suis is a major swine pathogen responsible for important economic losses to the swine industry worldwide. It is also an emerging zoonotic agent of meningitis and streptococcal toxic shock-like syndrome. Since the recent recognition of the high prevalence of *S. suis* human disease in southeast and east Asia, the interest of the scientific community in this pathogen has significantly increased. In the last few years, as a direct consequence of these intensified research efforts, large amounts of data on putative virulence factors have appeared in the literature. Although the presence of some proposed virulence factors does not necessarily define a *S. suis* strain as being virulent, several cell-associated or secreted factors are clearly important for the pathogenesis of the *S. suis* infection. In order to cause disease, *S. suis* must colonize the host, breach epithelial barriers, reach and survive in the bloodstream, invade different organs, and cause exaggerated inflammation. In this review, we discuss the potential contribution of different described *S. suis* virulence factors at each step of the pathogenesis of the infection. Finally, we briefly discuss other described virulence factors, virulence factor candidates and virulence markers for which a precise role at specific steps of the pathogenesis of the *S. suis* infection has not yet been clearly established.

Streptococcus suis is a major swine pathogen responsible for important economic losses to the porcine industry worldwide. It causes a wide variety of diseases in pigs, including, but not limited to, meningitis, septicemia and endocarditis [1]. Among the 35 serotypes based on capsular antigens that have been described, serotype 2 is the most frequently isolated from diseased pigs in most countries [1,2]. *S. suis*, especially the serotype 2, is also a zoonotic agent. First reported in Denmark in 1968 [3], human cases of *S. suis* infections have been documented in several European and Asian countries as well as in North and South America, Australia and New Zealand [4,5]. In western countries, *S. suis* infections in humans have most often been restricted to workers in close contact with pigs or swine byproducts. However, in southeast and east Asia, this bacterium also affects the general population and it represents a significant public health concern [4]. Two deadly human *S. suis* outbreaks occurred in China in 1998 and 2005 [6]. In addition, it has been shown that *S. suis* is the first cause of adult meningitis in Vietnam, the second in Thailand and the third most common cause of community-acquired bacterial meningitis in Hong Kong [7–10].

For infections in humans, readers are referred to two recent and comprehensive review articles [4,5]. We note, however, that the emergence of *S. suis* as a human agent of disease has led to an explosion of research on this pathogen. A search in PubMed with the keywords '*Streptococcus suis*' shows that of the 917 articles recorded in that database as of 31 August 2011, 339 were published during the last 5 years and more than 140 in the last 18 months. Since 2006, complete genome sequences of virulent and avirulent strains of different geographic origins have either been published [11–16], or are available in public

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databases. Moreover, in recent years, genetic tools such as *in vivo* expression technology, signature tagged mutagenesis, selective capture of transcribed sequences and suppression subtractive hybridization have been used to uncover the virulence arsenal of this organism [17–21]. In a recent article, Baums and Valentin-Weigand reviewed the surface-associated and secreted virulence factors of *S. suis* [22]. Here, we discuss the role of these, other and newly described virulence factors during the pathogenesis of the *S. suis* infection.

Identification of *S. suis* virulence factors has suffered from the lack of a clear definition of 'virulence'. Indeed, the concept may differ from one group of investigators to another. In addition, experimental infection models for *S. suis* can be misleading. These differences may explain some discrepancies regarding the virulence of a single strain of *S. suis* [23–25]. For example, different studies have defined field strains as virulent or avirulent based on the clinical condition of the animal (or human being) from which the strain was isolated (isolation from clinically healthy animals gives strains arbitrarily considered avirulent whereas isolation from diseased animals/human beings gives strains arbitrarily considered virulent); the presence of previously described and nonuniversal virulence-associated proteins; or the use of different experimental infection models (e.g., different strains of inbred or outbred mice; zebrafish; rabbits; the amoeba *Dictyostelium discoideum*; guinea pigs or the natural host, pigs, including mini-pigs, conventional pigs, specific-pathogen-free or colostrum-deprived piglets [pretreated or not with other microorganisms or respiratory irritants]) [26–34]. Furthermore, the presence of some proposed virulence factors does not necessarily define the strain as virulent. Indeed, using the same experimental swine infection model (see below), some strains possessing a putative virulence factor were shown to be avirulent, while other strains devoid of the same factor were still virulent [25]. Finally, the virulence of serotype 2 strains recovered in Europe and Asia from diseased piglets seems to be higher than that observed in strains from north America [25,35]. Despite these concerns, several cell-associated or secreted factors may clearly be considered as important for the pathogenesis of the *S. suis* infection. Table 1 summarizes most *S. suis* virulence factors proposed so far.

Overview of the pathogenesis of *S. suis* infection

Pigs may acquire *S. suis* via both vertical and horizontal transmission [1]. Colonized animals typically harbor the organism in their tonsils and may never develop disease (carrier animals) [36]. Conversely, some carrier piglets will eventually develop bacteremia, septicemia and/or meningitis due to dissemination of *S. suis* from tonsils and/or other mucosal surfaces, usually when maternal antibodies decline [37]. More specifically, to cause disease, bacteria must breach epithelial barriers, reach and survive in the bloodstream, invade different organs and cause exaggerated inflammation [1,22]. In humans, it is believed that individuals can become infected through skin lesions or the oral route [4], although carriage of *S. suis* by humans without clinical signs (usually abattoir workers) has also been described [4,38]. Infection of humans may also begin by colonization followed by invasion, bacteremia and septicemia with or without meningitis. In the following sections, we discuss the potential contribution of long established and recently described virulence factor candidates at each step of the pathogenesis of the infection. Finally, we briefly discuss other described virulence factors, virulence factor candidates and 'virulence markers' for which a precise role at specific steps of the pathogenesis of the *S. suis* infection has not yet been clearly established.

Colonization: adherence & invasion of epithelial surfaces

The actual early mechanisms used by *S. suis* to colonize the host are poorly known. The pathogen may survive in swine tonsils for long periods of time [Gottschalk M, Unpublished Data] [1]. The tonsillar lymphoid tissue is overlain by mucosal epithelium. In pigs, particularly, the palatine surface area is markedly increased by deep epithelial invaginations within the lymphoid tissue forming numerous branching crypts [39]. It is possible that after adhesion and invasion of epithelial cells in tonsils, bacteria remain hidden from the immune system. However, it is still unknown how *S. suis*, which is usually found in very low quantities in tonsils of pigs belonging to herds without clinical signs [37,40], manages to cross the first natural line of the host defense to initiate disease. The current most accepted hypothesis is that the pathogen breaches the mucosal epithelium in the upper respiratory tract of pigs [41]. Similarly, in humans, *S. suis* may interact with epithelial cells either at the epidermal surface or in the intestine (oral route of infection) [4,5,42]. Although bacterial adhesion and invasion of epithelial cells are not necessarily synonymous into colonization, they are usually associated with the very first steps of

Table 1. Confirmed and putative *Streptococcus suis* virulence factors.

Factor	Putative or confirmed function/role in virulence	Virulence of mutants defective for the factor in animal infection models (animal model)	Ref.
CpsE/F	CPS biosynthesis (glycosyltransferases)	Attenuated (pig)	[76,77,79]
Cps2C	CPS biosynthesis (chain length termination and export)	Attenuated (pig)	[19,79]
NeuB	*N*-acetylneuraminic acid (sialic acid) synthetase	Attenuated (pig)	[19,165]
PgdA	Peptidoglycan *N*-deacetylase	Attenuated (pig)	[88]
DltA	LTA D-alanylation	Attenuated (pig)	[89]
Suilysin	Hemolysin	Unaffected (pig)	[66,120,166, 167]
Muramidase-released protein	Unknown	Unaffected (pig)	[168,169]
Extracellular protein factor	Unknown	Unaffected (pig)	[157,169]
Fbps	Adhesin: fibronectin binding	Attenuated (pig)	[47]
Enolase	Adhesin: fibronectin and plaminogen binding	Mutant not available	[49]
Glyceraldehyde-3-phosphate dehydrogenase	Adhesin: plaminogen, porcine tracheal rings. Plasmin acquisition. Upregulated *in vivo*	Mutant not available	[54–56]
Dipeptidylpeptidase IV	Adhesin: fibronectin binding	Attenuated (mouse)	[52,170]
6-phosphogluconate-dehydrogenase	Adhesin (HEp-2 and HeLa cells)	Mutant not available	[59]
Amylopullulanase	Adhesin (porcine epithelium and mucus)	Not tested†	[61]
Glutamine synthetase	Adhesion to the epithelial cells HEp-2	Attenuated (mouse)	[60]
srtF pilus	Putative adhesin. In tested strains, the adhesin is not expressed	Unaffected (mouse)	[17,153,154]
srtG pilus	Putative adhesin	Not tested†	[153,155]
Adhesin P	Hemagglutinin	Mutant not available	[171]
Dpr	Resistance to iron-mediated toxicity	Mutant not available	[172,173]
Zur	Resistance to zinc-mediated toxicity	Not tested†	[174]
AdcR	Zinc uptake regulator	Attenuated (mouse)	[99]
Fur	Iron uptake regulator	Attenuated (mouse)	[99]
FeoB	Iron transporter	Attenuated (mouse)	[101]
TroA	Manganese uptake	Attenuated (mouse)	[104]
SSU0308 (Lipoprotein 103)	Zinc uptake	Attenuated (mouse)	[105]
Lipoprotein signal peptidase	Lipoprotein export	Unaffected (pig)	[175]
Lgt	Prolipoprotein diacylglyceryl transferase	Not tested†	[119]
lpp gene	Lipoprotein	Attenuated (pig)	[19]
Gene homologous to GBS SAG0907	Lipoprotein	Unaffected (pig)	[19]
Superoxide dismutase	Resistance to toxicity	Mutant not available	[106]
Arginine deiminase system	Resistance to acidity	Not tested†	[107,108]

†*Not tested indicates that a mutant strain defective in the production of the factor has been generated but its virulence not assessed in animal infection models.*
CPS: Capsular polysaccharide; Fbps: Fibronectin-fibrinogen binding protein; GBS: Group B Streptococcus*; LTA: Lipoteichoic acid.*

Table 1. Confirmed and putative *Streptococcus suis* virulence factors (cont.).

Factor	Putative or confirmed function/role in virulence	Virulence of mutants defective for the factor in animal infection models (animal model)	Ref.
SspA	Subtilisin-like protease	Attenuated (mouse)	[122,176,177]
LuxS	Quorum sensing	Attenuated (zebrafish)	[73,178]
IgA1 protease	IgA1 protease	Attenuated (pig)	[69,70]
Sortase A	Protein sorting	Attenuated (pig)	[53,179,180]
Serum opacity-like factor	Serum opacification	Attenuated (pig)	[142,181]
Endo-β-*N*-acetylglucosaminidase D	Degradation of host surface oligosaccharides	Attenuated (pig)	[19]
SsnA nuclease	Degradation of host DNA	Not tested†	[97]
Hyaluronate lyase	Degradation of hyaluronic acid	Not tested†	[182]
Collagenase	Degradation of collagen	Partly attenuated (pig)	[19]
Phospholipase C	Modulation of host arachidonic acid production	Mutant not available	[135]
SalK/SalR	Two-component signal transduction system	Attenuated (pig)	[100]
CiaRH	Two-component signal transduction system	Attenuated (pig)	[63]
RevSC21	Orphan response regulator	Attenuated (mouse)	[62]
CovR	Orphan response regulator	Hypervirulent (pig)	[64]
RevS	Orphan response regulator	Attenuated (mouse)	[144]
Rgg-like	Transcriptional regulator	Attenuated (pig)	[146]
treR gene	Transcriptional regulator	Attenuated (pig)	[19]
Gene homologous to *S. mutans* SMU_61	Transcriptional regulator	Attenuated (pig)	[19]
nadR gene	Transcriptional regulator	Attenuated (pig)	[19]
CcpA	Sugar catabolism regulator	Not tested†	[145]
scrR gene	Metabolism: sucrose operon repressor	Attenuated (pig)	[19]
38 KDa protein	Metabolism: putative phosphoglycerate mutase	Mutant not available	[183]
Glutamate dehydrogenase	Metabolism: glutamate dehydrogenase	Mutant not available	[184]
gtfA gene	Metabolism: sucrose phosphorylase	Attenuated (pig)	[19]
purA gene	Metabolism: adenylosuccinate synthetase	Attenuated (pig)	[19]
purD gene	Metabolism: phosphoribosylamine-glycine ligase	Attenuated (pig)	[19]
scrB gene	Metabolism: sucrose-6-phosphate hydrolase	Attenuated (pig)	[19]
cdd gene	Metabolism: cytidine deaminase	Attenuated (pig)	[19]
guaA gene	Metabolism: GMP synthase	Unaffected (pig)	[19]
guaB gene	Metabolism: inosinemonophosphatedehydrogenase	Unaffected (pig)	[19]
manN gene	Mannose-specific transport PTS IID	Attenuated (pig)	[19]
Permease	ABC-type multidrug transporter	Attenuated (pig)	[185]

†*Not tested indicates that a mutant strain defective in the production of the factor has been generated but its virulence not assessed in animal infection models.*
CPS: Capsular polysaccharide; Fbps: Fibronectin-fibrinogen binding protein; GBS: Group B Streptococcus*; LTA: Lipoteichoic acid.*

Table 1. Confirmed and putative *Streptococcus suis* virulence factors (cont.).

Factor	Putative or confirmed function/role in virulence	Virulence of mutants defective for the factor in animal infection models (animal model)	Ref.
Permease	ABC-type amino acid transporter	Attenuated (pig)	[19]
44 kDa membrane protein	Unknown	Not tested†	[186]
Gene homologous to *S. pneumoniae spr*1018	Unknown	Attenuated (pig)	[19]
glnH gene	Unknown	Attenuated (pig)	[19]
Surface antigen One	Unknown	Mutant not available	[151]
VirA	Unknown	Attenuated (rabbit)	[33]
Trag	Unknown	Attenuated (zebrafish)	[143]
Histidine triad protein Htps	Unknown	Unaffected (mouse)	[92,105]

†*Not tested indicates that a mutant strain defective in the production of the factor has been generated but its virulence not assessed in animal infection models.*
CPS: Capsular polysaccharide; Fbps: Fibronectin-fibrinogen binding protein; GBS: Group B Streptococcus*; LTA: Lipoteichoic acid.*

colonization by mucosal pathogens. Very few mechanistic studies are available regarding the interactions between *S. suis* and epithelial cells, with the exception of choroid plexus epithelial cells (CPEC; discussed in the 'CNS invasion and meningitis' section). It has been reported that virulent *S. suis* strains can adhere to epithelial cells from the respiratory tract of humans (Figure 1) [43–45]. Adhesins present at the surface of *S. suis* appear to be hindered by the capsular polysaccharide (CPS), as suggested by the fact that *S. suis* CPS-deficient mutants adhered better than the encapsulated parental strain to porcine (LLC-PK1 and PK15), canine (MDCK) and human (A549 and HeLa) cell lines. In addition, pretreatment of cells with purified *S. suis* cell wall material almost completely abrogated such adhesion [44]. Higher adhesion and invasion levels of human HEp-2 epithelial cells with unencapsulated strains were also reported by Benga *et al* [45]. Thus, it can be hypothesized that *S. suis* downregulates expression of CPS during the early steps of the infection in response to signals from the environment, resulting in a better interplay between bacterial adhesins and host receptors. This hypothesis needs to be explored.

S. suis interacts with components of the extracellular matrix (ECM) such as fibronectin and plasminogen (Figure 1) [46]. The fibronectin-binding protein Fbps was shown to bind human fibronectin and fibrinogen *in vitro* [47]. However, an isogenic *fbps* mutant did not have diminished binding to human fibronectin [46], suggesting that redundancy for binding to this ECM protein exists. Experimental infection of pigs with the *fbps* mutant strain showed that Fbps is not required for colonization of the tonsils (first steps of the infection) but that it may play a role in colonization of specific organs involved in *S. suis* infection [47]. Enolases at the surface of bacterial pathogens drive bacterial binding to plasminogen [48]. *S. suis* enolase was found to bind not only plasminogen but also fibronectin. Binding of enolase to fibronectin was an unprecedented finding. Using surface plasmon resonance, it was shown that the enolase–fibronectin interaction has low nanomolar affinity [49]. *S. suis* enolase is highly expressed *in vivo*, inducing the production of antibodies in infected pigs, although the potential for enolase being used as a protective antigen remains controversial [50,51]. Recently, a dipeptidylpeptidase DppIV expressed by *S. suis* was also shown to interact with human fibronectin; the virulence of a *dppIV*-deficient mutant was greatly attenuated [52]. Binding of *S. suis* to collagen has also been reported [46], and it has been shown that a mutant strain defective in a putative collagenase was severely impaired in survival after experimental inoculation of pigs [19]. A *S. suis* mutant strain devoid of the housekeeping sortase SrtA showed less adherence to ECM proteins [53], suggesting that peptidoglycan-anchored, LPXTG motif-containing adhesins are also important for interactions of this pathogen with ECM proteins.

Other *S. suis* proteins have also been identified as adhesins, including a 39-kDa glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [54,55]. Two different studies showed reduced adherence of *S. suis* to porcine tracheal rings and HEp-2 cells when cells were preincubated with recombinant *S. suis* GAPDH, suggesting the involvement of the protein in the first steps of bacterial

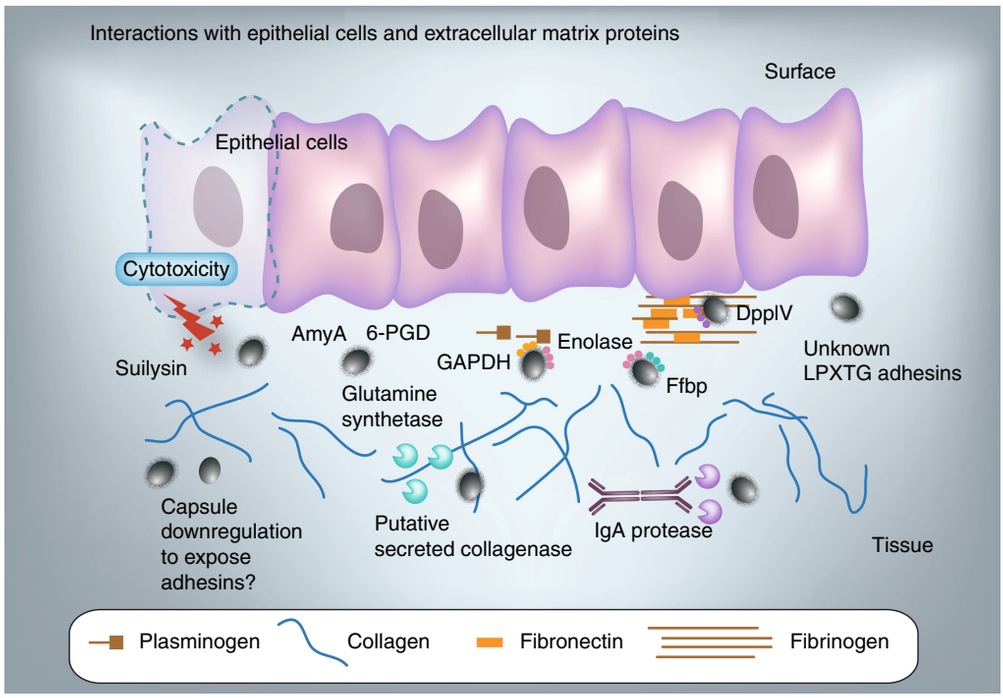

Figure 1. Interactions of *Streptococcus suis* with epithelial cells and extracellular matrix proteins. (For choroid plexus epithelial cells, see Figure 3). Adhesion of *Streptococcus suis* to epithelial cells is multifactorial. Examples of factors involved in adhesion are bacterial surface proteins such as enolase, GAPDH, 6-PGD, AmyA, as well as a glutamine synthetase. Unidentified LPXTG-containing adhesins (including pili) have been suggested to participate in adhesion to epithelial cells. The actual contribution of these factors has not yet been demonstrated. Invasion of epithelial cells other than choroid plexus epithelial cells by encapsulated *S. suis* serotype 2 is still controversial. In the case of suilysin-positive strains, expression of this hemolysin may be instrumental in breaching the epithelium. IgA protease-producing bacteria may also take advantage of released Fab fragments after IgA proteolysis to enhance their surface hydrophobicity and thus adhesion to host cells. *S. suis* binds extracellular matrix proteins such as fibronectin, plasminogen and collagen. Enolase, a fibronectin-fibrinogen binding protein, and a dipeptidylpeptidase IV all bind human fibronectin and fibrinogen. Enolase also mediates binding to plasminogen. Also necessary for bacterial-extracellular matrix proteins interactions are LPXTG-containing proteins, since deletion of SrtA impairs *S. suis* binding to these host factors. Collagen degradation by means of the secretion of a putative collagenase has been proposed. Capsule downregulation upon the interactions could facilitate the display of adhesins.
AmyA: Amylopullulanase A; 6-PGD: 6-phosphogluconate-dehydrogenase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

adhesion to host cells [55,56]. The contribution of GAPDH to the virulence of *S. suis* remains to be further characterized. However, it has been reported that *S. suis* highly upregulates the expression of the gene encoding this protein *in vivo* in different porcine organs [57]. In addition, GAPDH has been shown to be highly immunogenic in pigs [58].

Interestingly, many researchers have used epithelial cell adhesion as a model to evaluate virulence factors. An enzyme with 6-phosphogluconate-dehydrogenase activity, a bifunctional amylopullulanase, as well as a glutamine synthetase, were all shown to contribute to *S. suis* adherence to epithelial cells [59–61]. Mutants devoid of the two-component regulatory system CiaRH or the orphan transcriptional regulators RevSC21 and CovR were also impaired in adherence to epithelial cells [62–64]. However, the mechanisms for such reduced adherence have not yet been deciphered.

Invasion of epithelial cells may only be an additional step in colonization, without further consequences. However, epithelial cell invasion may also represent the beginning of systemic dissemination and disease. While cell invasion is one expected outcome of *S. suis* adhesion to epithelial cells, invasion of epithelial cells other than CPEC (see below) by well encapsulated *S. suis* serotype 2 is still controversial [44,45,65]. As in the case of adhesion, only unencapsulated strains seem to be able to invade these cells [65]. Epithelial cell disruption is also possible, since suilysin-positive strains are highly

toxic for these cells (Figure 1) [41]. This 54-kDa hemolysin is a thiol-activated toxin that targets cholesterol in the membrane of eukaryotic cells [66,67]. However, strains not producing suilysin are also able to reach the bloodstream and disseminate [1].

IgA-mediated immunity plays a major role in defense against recurrent mucosal pathogens. IgA protease-producing bacteria can overcome this defense by limiting the amount of functional IgA. They may also take advantage of released Fab fragments to enhance their surface hydrophobicity (and thus adhesion to host cells) and to block the access of intact antibodies (Figure 1) [68]. It has recently been reported that *S. suis* produces an IgA1 protease capable of efficiently cleaving human IgA1 [69]. The protease is highly immunoreactive to convalescent sera, and an isogenic mutant defective in the production of this enzyme showed significantly decreased lethality in pigs [70].

As mentioned, *S. suis* may colonize tonsils for a relatively long period of time. Many bacterial pathogens utilize a biofilm strategy to survive inhospitable conditions. A recent report has shown that *S. suis* is able to induce the production of biofilms [71] and additional data indicate that biofilm cells may present a lower virulence capacity than their planktonic counterparts [72]. Interestingly, *S. suis* bacterial cells found in biofilms were shown to downregulate the expression of genes involved in capsule biosynthesis [73] and Tanabe *et al.* described biofilms formed by unencapsulated strains [74]. However, it is not known whether *S. suis* produces biofilms when colonizing the tonsils or other organs.

Survival in blood & dissemination

As mentioned above, cell invasion or cell disruption may be considered the first step of systemic disease development. It has been proposed that *S. suis* may gain entry to the systemic circulation primarily through the palatine tonsils, after adhesion and invasion of epithelial cells and interaction with cells of the myeloid lineage [36,75]. Once *S. suis* reaches deep tissues and/or the bloodstream, it is subject to the action of phagocytic cells of the innate immune system. However, in the absence of specific antibodies, *S. suis* is able to resist phagocytosis and persist in blood at high concentrations, with inflammatory consequences that are discussed below. Bacterial survival largely depends on the production of CPS (Figure 2). It has been widely documented that the CPS protects *S. suis* from neutrophil and monocyte/macrophage-mediated phagocytosis and killing [1]. Indeed, several different *in vitro* and *in vivo* experiments using isogenic unencapsulated mutant strains have conclusively shown that the absence of CPS correlates with highly increased phagocytosis and/or killing of these strains by phagocytic cells, and a rapid clearance of the bacteria from circulation [76–79]. The fine structure of the *S. suis* serotype 2 CPS has recently been solved, indicating the presence of galactose (Gal), 6-linked Gal, 3, 4-linked Gal, 4-linked *N*-acetyl-glucosamine (GlcNAc), and 3, 4-linked rhamnose. *S. suis* CPS, similar to Group B *Streptococcus* (GBS), also contains *N*-acetyl-neuraminic acid (sialic acid) residues that are terminally linked to the CPS chain. However, while in GBS sialic acid is 2–3 linked to Gal, in *S. suis* the linkage is 2–6 to Gal [80]. Capsular sialic acid has been shown to be important in preventing the deposition of the complement protein C3 on the surface of GBS, therefore blocking activation of the alternative pathway and allowing for GBS resistance to opsonin-dependent intracellular killing [81]. Such a role has not yet been demonstrated for *S. suis* sialic acid. In fact, it has been so far very difficult to dissociate the effects of the sialic acid moiety from those of the whole CPS. We have recently produced a *neuC* isogenic mutant (*neuC* encodes an UDP-GlcNAc epimerase essential for sialic acid biosynthesis); however, deletion of *neuC* precludes the expression/assembly of the whole CPS, resulting in an unencapsulated phenotype [Gottschalk M, Unpublished Data]. It is worth noting that the capsules of the two most important serotypes that cause disease in humans (serotypes 2 and 14) possess sialic acid [82]. Strains of other serotypes whose capsules also contain sialic acid, such as serotypes 1 and 16, have occasionally induced human disease [83,84]. Sialic acid has also been implicated in the adherence (without phagocytosis) of *S. suis* to monocytes, suggesting a 'modified Trojan horse' hypothesis, in which the pathogen would travel in the bloodstream externally associated with these phagocytic cells (Figure 2) [41]. Finally, an effect of molecular mimicry has been suggested [1], based on the fact that the conserved 2–6 linked sialic acid terminal capsular moiety found in serotypes 2 and 14 [Gottschalk M, Unpublished Data] [80] is similar to sugar epitopes widely displayed on the surface of all mammalian cells [4]. This molecular mimicry could lead to absence of antigen recognition by the immune system of the host. In fact, *S. suis* serotype 2 CPS has been reported to be poorly immunogenic in both pigs and horses [85,86].

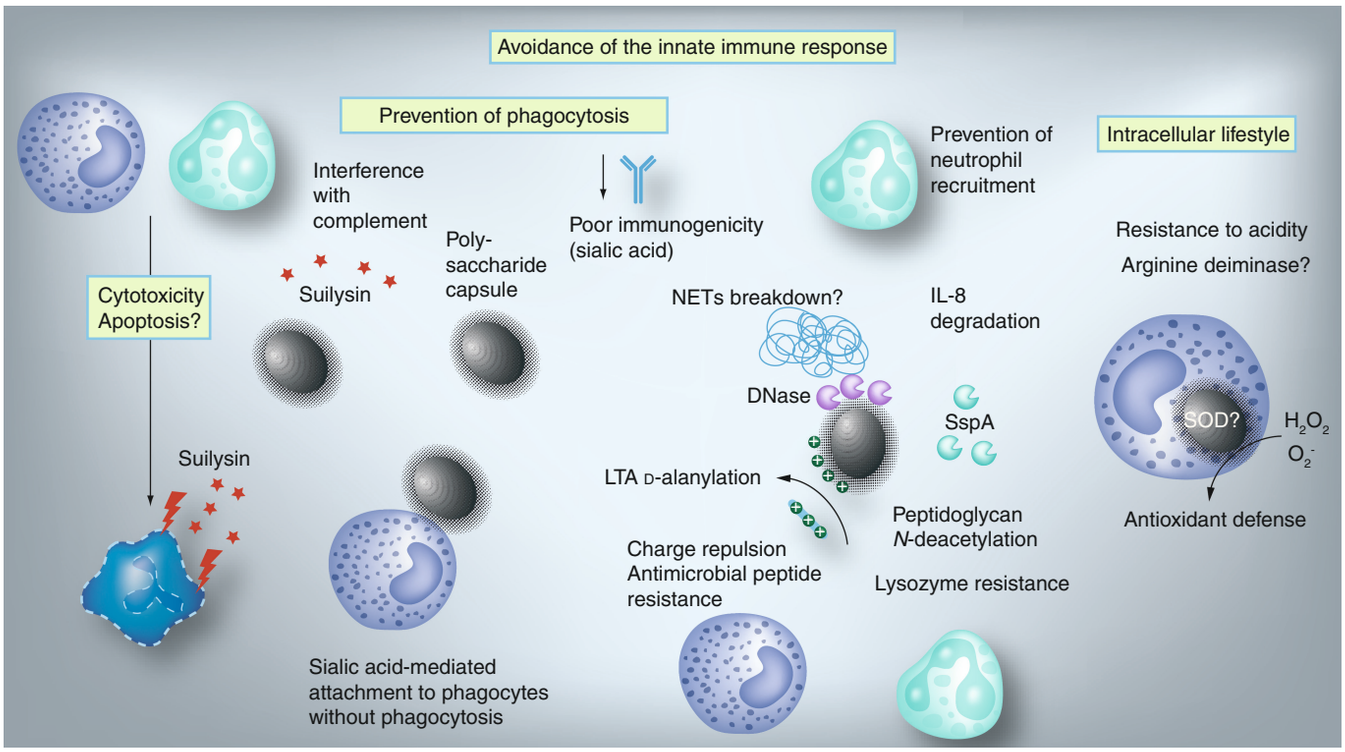

Figure 2. Avoidance of the host innate immune response. *Streptococcus suis* is an encapsulated organism. The polysaccharide capsule has been shown to interfere with and prevent phagocytocis and killing by host phagocytes. A suggested mechanism of immune avoidance is also the attachment to phagocytes, without phagocytosis. It is believed that this attachment is mediated by capsular sialic acid. *N*-deacetylation of the peptidoglycan also reduces killing by neutrophils, probably by providing the bacterium with enhanced resistance against the action of lysozyme. D-alanylation of the LTA contributes to enhanced resistance to host antimicrobial peptides and to resistance to neutrophil killing. *S. suis* produces a cell wall-anchored DNase, suggested to participate in NETs breakdown. The bacterium also secretes the serine protease SspA, capable of degrading IL-8, a major chemoattractant of neutrophils. Secreted suilysin has been shown to be toxic to phagocytes and also to interfere with complement activity. If internalized, *S. suis* may use SOD and the arginine deiminase system to resist the intracellular environment.
LTA: Lipoteichoic acid; NET: Neutrophil-extracellular trap; SOD: Superoxide dismutase.

Despite the critical role played by the CPS in *S. suis* virulence, the fact that a strain is encapsulated does not imply that the strain is virulent. Some avirulent serotype 2 field strains are well encapsulated [87], indicating that *S. suis* survival in blood does not rely solely on encapsulation (Figure 2). For instance, well encapsulated avirulent strains were eliminated from blood within 48 h, whereas a virulent strain could persist in circulation in relatively high titers for several days [1,25,27]. Resistance to phagocytosis is multifactorial and seems to also require modification of the cell wall peptidoglycan by means of *N*-deacetylation [88]. A well encapsulated mutant strain devoid of the deacetylase PgdA, responsible for this specific peptidoglycan modification, showed impaired resistance to neutrophil killing and was severely attenuated in murine and porcine infection models [88]. Interestingly, expression of the *pgdA* gene was highly upregulated by *S. suis* upon interaction with porcine neutrophils as well as *in vivo* in experimentally infected mice, suggesting that *S. suis* may enhance peptidoglycan *N*-deacetylation, and therefore better resist neutrophil lysozyme-mediated killing under these conditions [88]. Similarly, D-alanylation of the *S. suis* lipoteichoic acid (LTA) plays a major role in survival of this pathogen (Figure 2) [89]. Indeed, a mutant strain producing LTA devoid of D-alanine residues was more susceptible than the parental strain to the action of cationic antimicrobial peptides and killing by porcine neutrophils. It was also attenuated in murine and porcine infection models, probably as a consequence of its decreased ability to escape immune clearance mechanisms [89]. In addition to these major cell wall structures, many surface proteins have been shown to induce antibodies that increase the killing of *S. suis* by phagocytes [50,90–92]. However, the mechanisms of action of these proteins or their role at the bacterial-phagocyte interface are still unknown. Although suilysin-negative strains can be virulent and survive in blood, suilysin-positive strains seem to additionally

benefit from the toxic effects of this hemolysin for monocytes and neutrophils [76,93]. Moreover, suilysin appears to reduce phagocytosis and killing of *S. suis* (Figure 2) [76,94,95]. *S. suis* is also able to affect neutrophil recruitment by degrading IL-8, presumably by the production of a serine protease [96]. A cell wall-anchored DNase, specific for single- and double-stranded linear DNA is expressed by *S. suis* (Figure 2) [97]. The actual contribution of this DNase to the virulence of *S. suis* remains to be verified; we hypothesize that this DNase is likely to play a role in disruption of neutrophil extracellular traps [98].

S. suis requires nutrients including trace metals, whose availability within the infected host is relatively low. AdcR, a streptococcal transcription factor homologous to the zinc-uptake regulator Zur, and the ferric uptake regulator Fur, one of the most important transcription factors controlling iron metabolism, were both shown to be important for *S. suis* survival *in vivo* [99,100]. Also, a mutant strain devoid of the iron transporter FeoB was impaired in survival in a mouse infection model [101]. Using *in vivo* expression technology to uncover virulence candidates, 18 unique iron restriction-induced genes were identified, including the *cpsA* gene, encoding a putative regulator of CPS biosynthesis and *iri-7*, homolog of *Streptococcus mutans rpgG*, a gene involved in capsule biosynthesis [20]. It was proposed that because the CPS of *S. suis* becomes thicker after growth *in vivo*, where free iron is scarce, upregulated expression of *cps2A* and *rpgG* under iron starvation might be expected [20]. Although this is an attractive hypothesis, a clear link between iron starvation and CPS production remains to be verified. Using selective capture of transcribed sequences, other genes were shown to be upregulated upon iron restriction [18]; still there is no clear association between these genes and the capacity of *S. suis* to survive *in vivo*. Despite these findings, it has been suggested that iron availability may have little impact on *S. suis* growth. In fact, it has been reported that *S. suis*, which does not secrete siderophores, adapts to iron restricted conditions by a change in its metabolism, replacing iron by manganese or magnesium [102,103]. Interestingly, the lipoprotein TroA, which is required for *S. suis* growth in environments low in manganese, was shown to be crucial for bacterial survival *in vivo* [104]. Very recently, it was shown that deletion of a lipoprotein involved in zinc uptake resulted in a mutant strain that was 50-times less virulent than the parental strain [105].

As mentioned above, the CPS constitutes a physical barrier against phagocytosis. However, if internalized (most often in very low numbers), *S. suis* possesses factors that might contribute to resist the intracellular killing machinery of phagocytic cells [65]. Among them, it is worth mentioning the presence of an active superoxide dismutase (SodA), although it has been suggested that it is unlikely that that SodA produced by *S. suis* type 2 mediates intracellular survival of pathogenic isolates in macrophages [106]. In addition, survival of *S. suis* under acidic conditions has been linked to the presence of an arginine deiminase system catalyzing the conversion of arginine to ornithine, ammonia and carbon dioxide (Figure 2) [107,108].

Inflammatory activation & septic shock

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 [109]. Such an unbalanced reaction may harm the host through an unregulated release of endogenously generated inflammatory compounds. As evidenced by human outbreaks of toxic shock-like syndrome as well as by septic shock cases in Europe and Asia caused by *S. suis* (with short incubation time, rapid disease progression and a high rate of mortality), an important release of proinflammatory mediators is thought to take place during *S. suis* systemic infections [4]. Thus, the ability of *S. suis* to induce cytokine production may have considerable biological relevance. It has previously been demonstrated that *S. suis* serotype 2 virulent strains are able to induce the production of different proinflammatory cytokines by porcine, murine and human cells [78,93,110,111]. This was confirmed *in vivo* with a standardized mouse model of *S. suis* early septic shock and late meningitis/encephalitis [112]. The high levels of systemic cytokines TNF-α, IL-6 and -12, IFN-γ and the chemoattractants CCL2/MCP-1, CXCL1/KC, and CCL5/RANTES observed *in vivo* within 24 h postinfection are thought to be responsible for the sudden early death of animals. The regulatory cytokine IL-10 was upregulated following the onset of most proinflammatory cytokines, indicating a negative feedback mechanism to control the extent of the inflammatory response. Increased or reduced rates of septic shock were observed in *S. suis*-infected mice treated either with neutralizing antibodies against IL-10 or with recombinant IL-10, respectively [113]. These observations in mice may be

extended to pigs and humans, where high levels of proinflammatory cytokines and chemokines were detected in acutely infected individuals [12,96]. *In vitro* studies showed that some pattern-recognition receptors, such as CD14 and Toll-like receptor (TLR)-2, might be responsible for cell activation by *S. suis*, which would lead to release of inflammatory compounds [93,114–116]. However, using macrophages isolated from TLR2 knockout mice, a highly reduced, but not completely abrogated cytokine production was observed, a finding that suggests the involvement of other TLRs in cytokine production [114]. This was confirmed by *in vivo* experiments, which showed that TLR2 knockout mice were almost as susceptible to septic shock as wild-type mice [Gottschalk M, Unpublished Data].

Which are the virulence factors of *S. suis* responsible for such an exacerbated inflammatory activation? Most bacterial factors involved remain unknown. Many studies with unencapsulated mutants clearly pointed out bacterial cell wall components as being the major cytokine-inducing factors but the mechanisms remain obscure [74,78,93,95,110,111]. Data have been obtained indicating either clear activation (human and porcine phagoctyes) or no activation (human epithelial transfected cells) of cell receptors (TLR2) by whole bacterial suspensions of *S. suis* [112,114,117,118]. The use of different cell types may probably explain these controversial results. Lipoproteins present in the cell wall could be, at least in part, responsible for cell receptor(s) recognition [117]. Very recently, it has been shown that a putative prolipoprotein diacylglyceryl transferase present in *S. suis* cell wall is required for innate immune activation [119]. However, other factors can also contribute to inflammation. For example, the CPS specifically induces MCP-1 production in a MyD88-independent manner [95,114,115]. Suilysin was shown to activate phagocytes and to induce the release of proinflammatory cytokines [115,120]. In addition, suilysin might release hemoglobin from red blood cells, contributing to raise the levels of proinflammatory mediators by acting in synergy with *S. suis* cell wall components [121]. A surface-associated subtilisin-like protease (SspA) has recently been shown to induce the secretion of different proinflammatory cytokines and chemokines by macrophages [122]. Interestingly, a low concentration of SspA was associated with secretion of high amounts of CCL5, whereas the use of the same protein at high concentrations resulted in low amounts of CCL5 being detected, likely due to a proteolytic degradation of that chemokine by the same SspA (Figure 2) [122]. Similar results had been observed by Vanier *et al.* [96], who suggested that *S. suis* can induce an exacerbated release of inflammatory mediators resulting in massive recruitment of leukocytes and subsequent release of inflammatory mediators; however, *S. suis* may modulate this response, and improve its survival, by actively degrading the chemokines and thus delaying recruitment of neutrophils to the site of inflammation [96].

CNS invasion & meningitis

If death from sepsis or toxic shock-like syndrome does not occur, but the level of bacteremia remains high, *S. suis* may cause meningitis [25,27]. Of note, in some cases bacteremia may be unapparent and meningitis can present suddenly. As other blood-borne pathogens, *S. suis* must cross the blood–brain barrier (BBB) and/or the blood–cerebrospinal fluid (CSF)-barrier in order to cause CNS infections. 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 [123]. The main cellular type of the BBB is brain microvascular endothelial cells (BMEC). Adhesion to, but not invasion of human BMEC has been demonstrated for *S. suis* [124]. Although bacterial factors involved in adhesion have not been fully elucidated, participation of the CPS is considered unlikely [125]. Conversely, the pathogen proved able not only to attach, but also to invade immortalized porcine BMEC (Figure 3), as demonstrated by antibiotic protection assays and electron microscopy [126]. *S. suis* survived up to 7 h within porcine BMEC [126], which is an interesting finding since a crucial element for the development of meningitis is the ability of pathogens to cross the BBB as live bacteria [127]. Using the same cell line, Benga *et al.* also showed internalization of *S. suis*. However, these authors did not consider the number of internalized bacteria to be significant and reported inability of *S. suis* to invade porcine BMEC [128]. There are no general criteria to designate bacterial strains as invasive or not on the basis of the number of internalized bacteria, which may have led to different conclusions. In fact, some researchers have arbitrarily defined a threshold to define the event as bacterial invasion [128]. Vanier *et al.* also showed bacterial invasion of primary porcine BMEC [129].

As suggested for epithelial cells, the CPS of *S. suis* partially interferes with the adhesion/

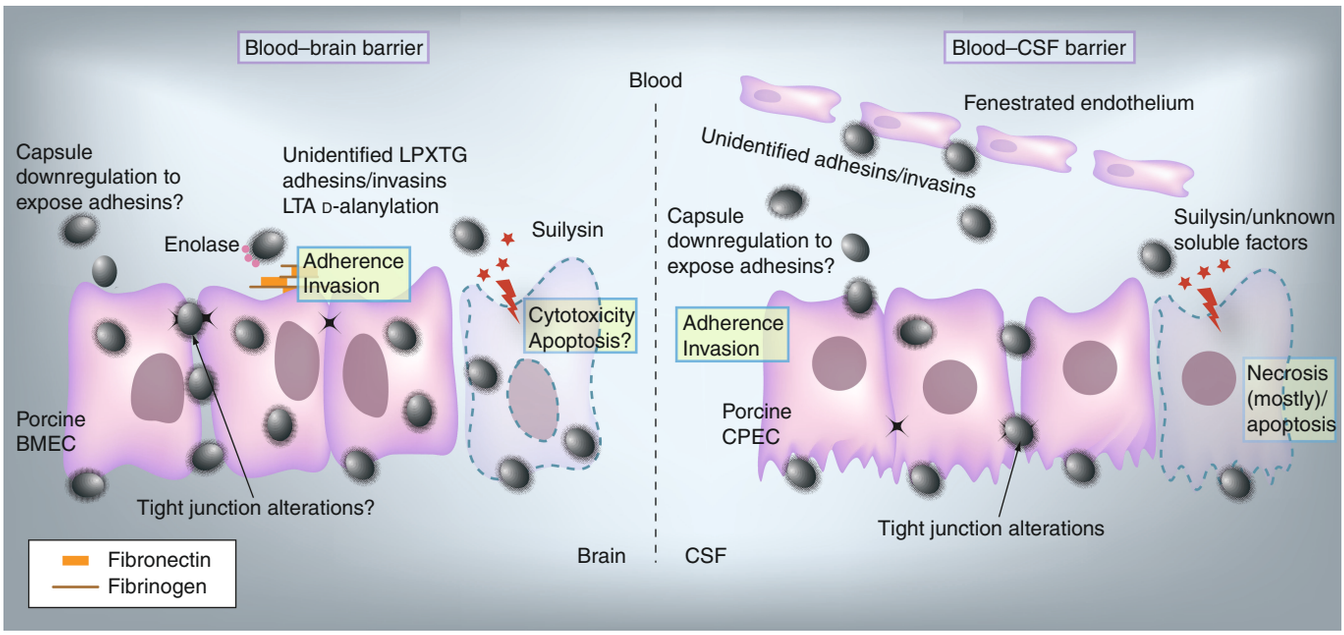

Figure 3. Invasion of the CNS. The two main CNS entry portals are the blood–brain barrier (BBB) and the blood–CSF barrier. The main cellular type of the BBB is BMEC. Invasion to and invasion of porcine BMEC is dependent on proteinaceous adhesins/invasins, and cell wall components such as the lipoteichoic acid (including lipoteichoic acid D-alanylation). Interaction with host extracellular matrix proteins (such as fibronectin/fibrinogen) may also be important. As suggested for epithelial cells, since the bacterial capsule partially interferes with the adhesion/invasion abilities of the pathogen; a possible downregulation of capsular polysaccharide expression has been suggested. LPXTG, cell wall-anchored proteins and enolase (through adhesion to fibronectin) may play a role as adhesins/invasins. Suilysin-positive strains may also disrupt the BBB through cytotoxic effects. Invasion and translocation of *Streptococcus suis* across the blood–CSF barrier has been shown. *S. suis* adhered better to porcine CPEC when applied to basolateral membranes, suggesting that direct access to the extracellular matrix was required. The capsular polysaccharide clearly compromised bacterial CPEC invasion, as demonstrated by the use of unencapsulated mutants, and indicating that bacterial cell wall components and/or surface proteins are needed. However, the nature of these adhesins/invasins remains largely unknown. *S. suis* is also able to affect the blood–CSF barrier function and integrity further facilitating trafficking of bacteria and leukocytes. It has been shown that *S. suis* induce CPEC necrosis, although apoptosis might also play a role in the process of cell death. Although other soluble factors might also be involved, suilysin plays an important role as a toxin affecting the blood–CSF barrier function.
BMEC: Brain microvascular endothelial cell; CPEC: Choroid plexus epithelial cell; CSF: Cerebrospinal fluid.

invasion abilities of the pathogen, perhaps because it hinders the display of putative adhesins (Figure 3) [74,126,128]. Further characterization of the invasion process suggested the involvement of proteinaceous adhesins/invasins and cell wall components such as the LTA [89,129]. Mutants impaired in LTA D-alanylation adhered and invaded porcine BMEC to a significantly lesser extent than the wild-type strain [89]. A *S. suis* SrtA mutant strain had reduced capacity to adhere and invade these cells, suggesting that LPXTG cell wall-anchored proteins may also serve as adhesins/invasins [53]. Serum components may also participate in the interactions between *S. suis* and porcine BMECs [126,128]. Among them, only fibronectin was shown to play an important role [129]; in addition, antibodies against enolase (an important fibronectin-binding protein in *S. suis*) significantly decreased adhesion and invasion of porcine BMEC [49]. Suilysin positive strains may also disrupt the BBB through cytotoxic effects, since at high bacterial doses suilysin-positive strains were toxic for porcine BMEC (Figure 3). However, suilysin was not indispensable for invasion, as a suilysin-negative mutant successfully invaded these cells [126].

Another CNS entry portal for *S. suis* may be the blood–CSF barrier CPECs (Figure 3). Indeed, although the blood–CSF barrier has a smaller surface area than the BBB, it may play an important role in bacterial translocation as well as in leukocyte transmigration. Recently, *in vitro* invasion and translocation of *S. suis* across the blood–CSF barrier (inverted transwell model) was shown [130]. This invasion was suggested to involve three potential steps: invasion of porcine CPEC from the basolateral side; transport within membrane-bound endocytic vacuoles to the apical side; and exocytosis onto the apical membrane of the blood–CSF barrier [130]. *S. suis* adhered and invaded cells better when applied to basolateral membranes

of porcine CPEC, suggesting that direct access to the ECM was required. The CPS clearly compromised bacterial CPEC invasion, as demonstrated by the use of unencapsulated mutants, and indicating that bacterial cell wall components and/or surface proteins are needed [130]. Very recently, using the same experimental transwell model, translocation across the blood–CSF barrier of *S. suis* activated neutrophils was demonstrated [131]. *S. suis* is also able to affect the blood–CSF barrier function and integrity, further facilitating trafficking of bacteria and leukocytes. It has been shown that *S. suis* induces CPEC necrosis, although apoptosis might also play a role in the process of cell death [132]. Interestingly, some isolates were able to disrupt the blood–CSF barrier when they were applied to the apical or to the basolateral compartment, signifying that soluble virulence factors in addition to direct bacteria–cell contact may alter the tightness of the porcine CPEC barrier [133]. It seems that suilysin plays an important role as a toxin affecting the blood–CSF barrier function; however, other soluble factors may also be involved [130,133].

Meningitis-associated brain injury and neuronal death is also associated with a host reaction to bacterial components [134]. *In vivo* experiments showed that infected mice who survive the septicemic phase can develop serious signs of inflammation at the CNS [112]. *In situ* hybridization of sampled brains showed transcriptional activation of TLRs, as well as activation of different inflammatory mediators. It has been shown that *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 [135]. It may also upregulate the expression of adhesion molecules on human monocytes and endothelial cells with consequent increased adherence of *S. suis*-activated monocytes [136,137]. Other studies have shown that *S. suis* is able to induce the release of proinflammatory cytokines and chemokines by human and porcine BMEC, murine microglia and astrocytes [112,118,138,139]. As indicated in the septic shock section, the specific bacterial components responsible for exaggerated inflammatory reactions are not accurately known. However, almost all *in vitro* studies mentioned above showed that unencapsulated mutant strains induce much higher levels of inflammatory mediators than the wild-type strains. However, recent studies using oligonucleotide microarray analysis and quantitative PCR surprisingly showed that adherent wild-type and capsule-deficient *S. suis* influenced the expression of a remarkably similar set of genes (including those involved in 'inflammatory response') in porcine CPEC [140]. In addition to cell wall components, bacterial CPS induces human macrophages to secrete prostaglandin E2 and matrix metalloproteinase 9, which may also be involved in disruption of the BBB [141]. Finally, purified suilysin has been shown to induce the release of several proinflammatory cytokines by human and porcine BMEC [96,126,138] and the upregulation of adhesion molecules on human monocytes [136].

Putative virulence factors & virulence markers with unknown or poorly defined functions in the pathogenesis of the infection

Many *S. suis* virulence factors have been shown to play specific roles at one or more steps of the pathogenesis of the infection. For many other factors, however, a clear association with a specific role in the development of disease has not been found, despite the fact that absence of the factor (demonstrated either using knockout mutants or neutralization of the factor by specific antibodies) affects virulence. In fact, many genes encoding homologs of known virulence factors in other Gram-positive organisms have been targeted for mutagenesis in *S. suis* and shown to contribute to its virulence. Examples of these virulence factors are a glutamine synthetase [60], a serum opacity-like factor [142], a protein of unknown function encoded by *virA* [33] and a trag factor [143]. As mentioned, the mechanisms by which these factors affect *S. suis* virulence remain obscure. A similar strategy, involving mutagenesis of genes homologous to global regulators of other Gram-positive bacteria, has identified a variety of proteins whose deletion affect virulence, including the response regulator RevS [144], the autoinducer LuxS [73], the sugar regulator CcpA [145], Rgg-like regulators [146], the orphan transcriptional regulators RevSC21 [62] and CovR [64], as well as the SalK/SalR [100] and CiaRH two-component systems [63]. Again, how these regulators influence virulence (and the extent of their regulons) has not yet been elucidated. Finally, although virulence factors and protective antigens are not necessarily the same, other proteins have been suspected to be 'virulence factors' based on the fact that antibodies against them confer protection. Examples are HP0245, HP0272 and HP0197,

of unknown function [147–150], the surface antigen One [151], and HtpS, a histidine triad protein [92]. However, recently obtained results showed that an isogenic mutant of *S. suis* lacking surface antigen One protein is as virulent as its wild-type strain [Gottschalk M, Unpublished Data]. Similarly, Aranda *et al.* did not observe differences in virulence when they compared an Htps mutant to its parental strain in a mouse infection model [105].

Other factors that clearly participate in the virulence of other pathogens could not be associated with virulence in *S. suis*. One of the best examples are pili, which have lately been shown to be important for the virulence of many *Streptococcus* species [152]. Homology database searches identified at least four putative pilus-encoding gene clusters in *S. suis* [17,153]. One of them, named the *srtF* cluster, is a truncated homologue of GBS pilus island 2b, composed of a signal peptidase-like and a class C sortase-encoding genes, and two genes encoding an ancillary and the major pilin subunits [17,153,154]. It has recently been demonstrated that *S. suis* express pili from this cluster, although the pili were formed by the major pilin subunit only, due to nonsense mutations at the 5′ end of the gene encoding the ancillary subunit (a putative adhesin) [154]. Abolishment of the expression of *srtF* cluster-encoded pili did not result in impaired interactions of *S. suis* with porcine BMEC. Furthermore, nonpiliated mutants were as virulent as the parent strain when evaluated in a murine model of *S. suis* sepsis. More recently, a second pilus, encoded by the *srtG* cluster, was shown to be expressed by some *S. suis* serotype 2 strains [155]. Interestingly, this pilus was expressed at higher levels when bacteria were grown at temperatures less than 30°C. It has been reported that surface temperatures of different external body parts of pigs (snout, ears, vertex, back and flank) range from 20 to 30°C when the environmental temperature is approximately 20°C [155]. These findings suggest that this pilus could be important for the interactions of *S. suis* with surface structures of host animals [155]. This hypothesis remains to be confirmed.

Some bacterial factors have been extensively used to try to predict the virulence of *S. suis* strains. They are considered virulence markers rather than 'virulence factors' since mutants devoid of such factors were shown to be as virulent as their respective parental strains. In addition, there are no hints on a potential role of such factors in the pathogenesis of the infection. For example, two proteins known as MRP and EF protein, respectively, have historically been used as virulence markers [156]. MRP is a 136-kDa cell-wall anchored protein also released into the culture supernatant during bacterial growth, while EF is a 110-kDa secreted protein [156]. Both proteins have variants of different molecular weights [157,158]. It was suggested that the production of these proteins may only be coincidentally associated with virulence rather than being actual virulence factors [1]. Although an association of MRP and EF with virulence is observed with strains from certain countries, the absence of one or more of these proteins does not necessarily result in lack of virulence. For example, in north America, a MRP^+ strain was reported to be avirulent while a MRP^- strain was shown to be virulent [25,159]. It is also possible that MRP^+ EF^+ strains might be potentially more virulent than MRP^- EF^- strains [1].

Conclusion

S. suis is an important swine pathogen and an emerging zoonotic agent. Our understanding of the pathogenesis of the infection in swine, as well as that of the two main clinical presentations observed in humans, septicemia with septic shock and meningitis, have tremendously improved in recent years. However, it is still difficult to associate all virulence factors proposed in the last few years with different steps of the infectious process. For some virulence factors, *in vivo* regulation may play an important role. For example, overexpression of the sialic-acid rich capsule in the bloodstream is required to avoid phagocytosis and killing by innate immune cells, but the pathogen needs to reduce capsule expression to avoid hindering adhesins important for attachment to epithelial/endothelial cells and formation of biofilms. In fact, it has been very recently found that unencapsulated *S. suis* field strains showed increased adherence to porcine and human platelets, a major virulence determinant for infective endocarditis. Interestingly, 34% of isolates recovered from this type of infection were unencapsulated [160]. Other factors, such as suilysin, may be important for virulence, but are certainly not essential. Suilysin is toxic for different cell types, affects complement, increases the BBB and blood–CSF barrier permeability and induces an inflammatory reaction. However, it is dispensable for full virulence and is not produced by most virulent strains isolated in north America [1]. Although many virulence factors have been suggested,

once a strain is isolated from a healthy carrier, there is no validated method to predict whether the strain is potentially virulent. Some virulence markers (without clear explanation of their roles in the pathogenesis of the infection) might be used in some parts of the world (MRP and EF). However, it is still impossible to ensure that a strain without these factors is indeed avirulent. In any case, results obtained during the last decade clearly indicate that strains from different geographical areas possess different virulence factors and different virulence potential. These results also clearly show that there is probably not a universal virulence factor for all *S. suis* strains. As shown in this review, this bacterium is a good example of a pathogen with multifactorial (and sometimes redundant) virulence factors. At each step of the infection, *S. suis* deploys an arsenal of virulence factors, either secreted and/or located at the cell surface and/or the cell wall that play important functions in the pathogenesis of the infection.

Consequently, studying the importance of virulence factors (as well as protective antigens) in *S. suis* is a challenging task. In fact, during the last 5 years, many proteins have been classified as critical for virulence through the use of gene knockout mutants, obtained either by transposon insertion or by allelic replacement. The role of a few of them in virulence can easily be deduced by the fact that they are regulatory genes influencing the expression of many other genes that are, in some cases, also putative virulence factors. The involvement in *S. suis* pathogenesis of other genes described as virulence factors is also easily understood, since these deleted genes are indispensable enzymes of known metabolic pathways. However, some factors have been described as critical for virulence based on abolishment or impairment of virulence after deletion of the factor-encoding gene, but the mechanism(s) of action of these factors have not been elucidated or, in some cases, not even a hypothetical mode of action proposed. More studies are necessary to confirm their role as significant virulence factors and to genuinely understand the mechanisms by which they contribute to the pathogenesis of the infection. It is possible that the definition of 'critical' virulence factor may also be influenced by the variety of animal infection models (including different routes of inoculation) used in the last few years. The role of the host in the interactions with different bacterial virulence candidates should also be taken into consideration.

Although this review did not concentrate in vaccination, it is worth mentioning that after more than 30 years of research, there is still no proven and commercially available subunit vaccine (based on well identified virulence factors or protective antigens) for use in swine to fight against the infection caused by *S. suis*. Being a pathogen with multifactorial virulence factors, these findings are to a certain extent expected. Surprisingly, different single antigens included in many vaccination trials during the last few years appeared to be highly protective. The methodology to measure protection against the infection caused by *S. suis* is far from being standardized, and conflicting results can be obtained with the same vaccine candidate [51,161]. Despite these pitfalls, the interest of the scientific community in this pathogen during the last few years has amazingly increased. Recent research has brought a colossal amount of novel information that is contributing to elucidate the pathogenesis of the infection caused by *S. suis*.

Future perspective

There is a clear need for standardization of animal models to study both the role of well identified virulence factors and protective antigens. It is critical to test in parallel, under the same methodological conditions, different avirulent mutants, meticulously shown to grow at similar rates to their respective wild-type parent strains. Similar studies can be carried out with protective antigens. It may be advantageous for the scientific community studying *S. suis*, which has significantly enlarged after the human outbreaks in Asia, to work in close collaboration in order to obtain validated results.

The role of some identified virulence factors needs to be further studied. One important factor is the CPS. Results obtained so far suggest that some of the genes responsible for capsule expression are differentially regulated at different steps of the pathogenesis of the infection. Future work on regulatory genes and quorum sensing will undoubtedly provide new and exciting evidence on how this pathogen causes disease. Future work may also need to be directed towards the confirmation of differences in virulence between strains isolated from different geographical regions. Although, as very recently reported, *S. suis* serotype 2 strains circulating in north America are less virulent [35,162], they still cause important economic losses to the swine industry, and human cases have now been described. *S. suis* serotype 2 strains different from the typical Eurasian

virulent strains, and previously described as 'less virulent types' have been found in several cases of *S. suis* disease in humans in Thailand [10,163,164]. More attention should be paid to these 'less virulent' strains.

Finally, although serotype 2 is still the serotype most frequently associated with disease, other capsular types have arisen in the last few years as pathogens, namely serotypes 1, 14, 16 and, more recently, serotypes 5 and 24 [42]. Full-genome sequence data is now available for several serotype 2 strains, one serotype 14 strain and one serotype 3 strain [11–16]. Full-genome sequencing of other strains of these and other serotypes will be instrumental in the identification of the molecular basis of virulence differences observed between the different serotypes and among strains within the same serotype. Although it will not be easy to establish animal models for each serotype, data on virulence factors and the pathogenesis of the infection caused by these more atypical serotypes will add valuable information on how this swine pathogen and zoonotic agent causes disease.

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Executive summary

Before analyzing virulence factors, how to compare the virulence of different* Streptococcus suis *strains?

- There is no consensus on the animal model to be used to evaluate the virulence of a specific strain of *Streptococcus suis*. Well-standardized, reproducible models (e.g., reference virulent strains, animal species, route of inoculation and bacterial infective doses) should be adopted by the scientific community in order to compare results from different research groups.
- Similarly, results regarding protection by immunization with specific, previously identified virulence factors may be misleading. A standardized methodology should be defined and agreed upon by the scientific community and used to compare protection conferred by already described factors and those that need further investigation.
- Despite those limitations, research performed during the last 20 years has significantly improved our knowledge of the pathogenesis of the *S. suis* infection through the identification and characterization of different virulence factor candidates.

Before reaching the bloodstream, bacteria must breach epithelial surfaces

- *S. suis* is able to adhere to epithelial cells of the upper respiratory tract of pigs and humans. Different bacterial adhesins have been described. The capsular polysaccharide (CPS) hinders the display of many of those adhesins. Thus, capsule downregulation seems likely to occur when *S. suis* interacts with these cells. Some host proteins, such as fibronectin, play an important role in interactions between *S. suis* and cell surfaces, and different extracellular matrix-binding proteins have been described for this pathogen.
- Invasion of epithelial cells of the respiratory tract by *S. suis* is still controversial. Only unencapsulated strains have been shown to be able to invade such cells. However, recent results using a transwell system showed that *S. suis* is able to invade epithelial cells from the choroid plexus. *S. suis* might also use necrosis of cells through the production of suilysin (a hemolysin).

Survival in the bloodstream & the ability to cause an exaggerated inflammatory reaction

- *S. suis* survival in blood is central to disease. The presence of the CPS and modifications of cell wall components protect bacteria from phagocytosis. The presence of sialic acid in the CPS may also contribute to bacterial survival. Some toxic compounds, such as suilysin, have been shown to affect phagocytes and complement. *S. suis* seems to be well adapted to a hostile milieu, expressing different proteins that are able to recover trace metals.
- Virulent *S. suis* strains succeed in maintaining a high bacterial concentration in blood. Factors present mainly in the bacterial cell wall (such as lipoproteins) will then trigger an important release of host inflammatory mediators that can lead to a toxic shock-like syndrome.

Invasion of the CNS: inflammation again plays a key role

- After surviving in blood, *S. suis* is able to cross the blood–brain barrier and/or the blood–cerebrospinal fluid barrier. *S. suis* is able to adhere and, in some cases, invade brain microvascular endothelial cell/choroid plexus epithelial cells, through adhesins also present in the cell wall underneath the CPS. The toxic effect of suilysin (and other unidentified products) may also contribute to increase the permeability of the blood–brain barrier/blood–cerebrospinal fluid barrier. *S. suis* cell wall components (and to a much lesser extent, the CPS and suilysin) are also able to increase the expression of adhesion molecules and proinflammatory cytokines, increasing *in situ* inflammation. *S. suis* is also able to invade the CNS through the choroid plexus, through adhesion and invasion of epithelial cells.
- Once in the CNS, an exaggerated inflammatory reaction takes place through activation of microglial cells, astrocytes and possibly other cell types of the brain by cell wall and secreted (suilysin) components of *S. suis*. Inflammation of the CNS leads to intracranial complications, including brain edema, increased intracranial pressure and cerebrovascular insults.

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- ▪ of interest
- ▪▪ of considerable interest

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