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Surface-associated and secreted factors of *Streptococcus suis* in epidemiology, pathogenesis and vaccine development

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Abstract

Streptococcus suis is an invasive porcine pathogen associated with meningitis, arthritis, bronchopneumonia and other diseases. The pathogen constitutes a major health problem in the swine industry worldwide. Furthermore, *S. suis* is an important zoonotic agent causing meningitis and other diseases in humans exposed to pigs or pork. Current knowledge on pathogenesis is limited, despite the enormous amount of data generated by 'omics' research. Accordingly, immunprophylaxis (in pigs) is hampered by lack of a cross-protective vaccine against virulent strains of this diverse species. This review focuses on bacterial factors, both surface-associated and secreted ones, which are considered to contribute to *S. suis* interaction(s) with host factors and cells. Factors are presented with respect to (i) their identification and features, (ii) their distribution among *S. suis* and (iii) their significance for virulence, immune response and vaccination. This review also shows the enormous progress made in research on *S. suis* over the last few years, and it emphasizes the numerous challenging questions remaining to be answered in the future.

Keywords: capsule, PgdA, sortase, MRP, HylA, SAO, OFS, FBPS, ArcA, GAPDH, enolase, suilysin, EF

Introduction

Streptococcus suis is the most important bacterial cause of meningitis in pigs worldwide. Furthermore, various other organs might be infected by this pathogen leading to different pathologies such as arthritis, serositis, endocarditis, otitis media and bronchopneumonia. Typically, these inflammations are acute and fibrinosuppurative (Williams and Blakemore, 1990; Madsen *et al.*, 2002; Beineke *et al.*, 2008). *S. suis* causes diseases in pigs of different ages, including suckling and weaning piglets as well as growers. Importantly, *S. suis* is not only an invasive pathogen but also a very successful colonizer of mucosal surfaces, in particular the upper respiratory tract. Healthy carrier piglets are the major reservoir of this pathogen and key players in the epidemiology of *S. suis* diseases (Clifton-Hadley and Alexander, 1980).

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S. suis, in particular serotype 2 strains, might also cause meningitis, septicemia, endocarditis and other diseases in humans. Close contact with pigs, wild boars or pork is considered to be an important risk factor for this zoonosis (Arends and Zanen, 1988; Baums *et al.*, 2007). In Asia, *S. suis* is classified as an emerging zoonotic pathogen as it was involved in two large outbreaks of severe human infections in 1998 and 2005 in China, and has recently been identified as one of the most important causes of bacterial meningitis in adults (Mai *et al.*, 2008; Wertheim *et al.*, 2009). Importantly, streptococcal toxic shock-like syndrome (STSS) was observed in numerous cases in the two outbreaks in China (Tang *et al.*, 2006). As the outbreaks were associated with a specific sequence type (ST7), it was suggested that ST7 strains carry additional virulence traits responsible for STSS manifestation (Ye *et al.*, 2006; Chen *et al.*, 2007). Genome comparisons revealed a putative pathogenicity island of 89 kb (89K PAI) present in STSS strains 98HAH12 (Chinese outbreak 1998) and O5ZYH33 (Chinese outbreak 2005) but not in the

European serotype 2 reference strain P1/7. The 89K PAI carries putative virulence genes, such as those encoding a two-component signal transduction system involved in virulence (Chen *et al.*, 2007; Li *et al.*, 2008). However, this review does not focus on STSS. For further details on the zoonotic potential of *S. suis*, the reader is referred to excellent recent reviews (Gottschalk *et al.*, 2007; Lun *et al.*, 2007).

S. suis is a very heterogeneous species. Thirty-three serotypes have been identified so far. Serotype 2 is worldwide the most important one. In China, serotype 2 accounts for more than 70% of the systemic *S. suis* diseases in piglets (Wei *et al.*, 2009). However, important geographical differences have been documented with regard to prevalences of *S. suis* serotypes. In some European countries with large pig industries, such as The Netherlands and Germany, serotype 9 has emerged as the most common serotype (Wisselink *et al.*, 2000). In the United Kingdom, serotypes 1 and 14 were reported to play important roles in invasive *S. suis* diseases, predominantly in suckling piglets. Serotype 7 strains have been associated frequently with bronchopneumonia in Scandinavia and, to a lesser extent, in Germany (Perch *et al.*, 1983; Aarestrup *et al.*, 1998; Tian *et al.*, 2004; Silva *et al.*, 2006). In Canada and the United States, serotypes 2, 1/2 and 3 have been reported to be the most prevalent. Most of the clinical *S. suis* isolates in Canada were from the lungs of piglets (Galina *et al.*, 1996; Messier *et al.*, 2008).

This review focuses on surface-associated and secreted factors of *S. suis*, which have attracted substantial scientific attention during the last few years. Identification (and characterization) of these factors is important for understanding pathogenesis and also for development of a cross-protective vaccine. Therefore, this review describes various *S. suis* factors, first surface-associated and then secreted factors, with respect to their identification and features, their distribution among *S. suis*, and their possible significance for pathogenicity, immune response and vaccination. For a more general overview on *S. suis*, we recommend the review by Higgins and Gottschalk (2005).

Cell wall and surface-associated factors of *S. suis*

The capsule

Serotyping of *S. suis* is based on differences in composition and structure of the polysaccharide capsule. For *S. suis* serotype 2, it has been demonstrated by two independent laboratories that the capsule is an important virulence factor (Charland *et al.*, 1998; Smith *et al.*, 1999). However, unencapsulated *S. suis* strains might also invade host tissue, though to a lower degree. This was shown by isolation of isogenic unencapsulated mutants from serosa, joints and central nervous system after intranasal application (Smith *et al.*, 1999). Large differences in virulence have been described for strains belonging to the same serotype (Vecht *et al.*, 1992), indicating that the capsule of *S. suis* is not sufficient for full virulence and that other factors have important functions in the pathogenesis of *S. suis*.

The capsule of serotypes 1 and 2 strains contains N-acetyl neuraminic acid (sialic acid) and four additional sugars, namely glucose, galactose, N-acetyl glucosamine and N-acetyl galactosamine (serotype 1) or rhamnose (serotype 2) (Elliott and Tai, 1978). Smith *et al.* (1999) sequenced the *cps*2 locus and identified genes encoding putative glucosyl-, galactosyl-, N-acetylglucosaminyl- and rhamnosyl-transferases (genes *cps*E-K). Furthermore, strains of serotypes 1, 2, 14, 27 and 1/2 carry the genes involved in sialic acid synthesis (Smith *et al.*, 2000). In many bacteria, surface-associated sialic acid contributes to pathogenesis by interfering with the activation of the alternative complement pathway by increasing the affinity of C3b to factor H (relative to factor B). This prevents formation of the C3 convertase C3bBb thereby limiting C3b deposition on the surface of the pathogen (Marques *et al.*, 1992). In *S. suis*, sialic acid contributes to the adherence of serotype 2 strains to murine macrophages (Segura and Gottschalk, 2002). However, adherence was not accompanied by phagocytosis. This finding supports the 'modified Trojan horse theory', as proposed earlier by Gottschalk and Segura (2000). The 'modified Trojan horse theory' suggests that *S. suis* strains, at least suilysin negative strains (see below), may cross the blood–brain–barrier (BBB) through adherence to immune cells, in particular monocytes, which may function as vehicles for passage through the BBB. However, there is also evidence for invasion of choroid plexus epithelial cells and directed transport of *S. suis* through these cells from the basolateral to the apical site, which faces the cerebrospinal fluid compartment (Tenenbaum *et al.*, 2009).

In contrast to the wild-type, isogenic unencapsulated mutants of serotype 2 strains were efficiently eliminated by primary alveolar macrophages (Smith *et al.*, 1999) as well as by the murine macrophage cell line J774 (Segura *et al.*, 1998). Segura *et al.* (2004) found that, unlike the encapsulated wild-type strain, the unencapsulated mutant-induced phosphorylation of the phosphatidylinositol-3 kinase downstream kinases Akt and PKCα in J774 macrophages, indicating the importance of these signaling pathways in phagocytosis of unencapsulated *S. suis* strains. Furthermore, in macrophages deficient in a tyrosine phosphatase (SHP-1), phagocytosis of an encapsulated *S. suis* wild-type strain was significantly increased. This was associated with Akt phosphorylation, suggesting that capsule-mediated resistance to phagocytosis involves activation of SHP-1 (Segura *et al.*, 2004).

The capsule of *S. suis* might also have other important immunomodulatory functions. As cell wall components of *S. suis* induce a significant release of proinflammatory cytokines (tumor necrosis factor-α (TNF-α), IL-1β and IL6) and chemokines (IL-8 and monocyte chemoattractant protein 1 (MCP-1)) by host cells, the capsule might reduce

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the production of such cytokines by blocking the interaction of cell wall components with pattern-recognition receptors, most importantly Toll-like receptor (TLR)2. On the other hand, the capsule of *S. suis* serotype 2 itself induces macrophage MCP-1 release through a TLR2- and MyD88-independent pathway (Graveline *et al.*, 2007). The authors discussed induction of MCP-1 release as part of the mechanism used by *S. suis* to translocate the BBB, as brain microvascular endothelial cells (BMECs) were shown to alter expression of tight junction-associated proteins in response to MCP-1 *in vitro* (Song and Pachter, 2004).

Adhesion of encapsulated serotype 2 strains of *S. suis* to epithelial cells is generally low, with values below 5% (Lalonde *et al.*, 2000) or about 1% (Benga *et al.*, 2004). In contrast, unencapsulated strains, including isogenic *cps*2 mutants, show adherence of 15–60% (Benga *et al.*, 2004). Interestingly, capsule expression was reported to interfere with adhesion to BMECs of porcine (Vanier *et al.*, 2004) but not of human origin (Charland *et al.*, 2000). It was suggested that porcine, but not human BMECs, might express an important receptor molecule that is recognized by unencapsulated but not encapsulated *S. suis* strains (Vanier *et al.*, 2004). However, such a receptor has not been identified and, furthermore, in such a case one would expect lower adhesion values of the unencapsulated strains to human than to porcine BMECs.

Norton *et al.* (1999) described invasion of the epithelial cell line HEp2 by encapsulated *S. suis* serotype 2 strains, in contrast to observations published for various epithelial cell lines by other laboratories (Lalonde *et al.*, 2000; Benga *et al.*, 2004). However, unencapsulated *S. suis* strains, including an isogenic *cps*2 mutant, have been demonstrated to be invasive, most likely through receptor-mediated endocytosis. A plausible explanation for the different phenotypes of encapsulated and unencapsulated *S. suis* strains might be that *S. suis* down-regulates capsule expression for increased adhesion to epithelial cells and upregulates it for protection against phagocytosis as it enters the bloodstream (Gottschalk and Segura, 2000). Accordingly, an increased thickness of the capsule was demonstrated during cultivation of *S. suis* in liquid media supplemented with serum (Wibawan and Lammler, 1994). Furthermore, increased thickness of the capsule was also noted during growth in the intra-peritoneal cavities in rats and pigs (Quessy *et al.*, 1994; Charland *et al.*, 1996). Interestingly, Smith *et al.* (2001) identified an *in vivo*-induced gene that encodes a protein highly homologous to CpsY, a regulator of capsule expression in *Streptococcus agalactiae* (73% identity). Further evidence for capsule regulation in *S. suis* is provided by a recent study on the orphan response regulator CovR (Pan *et al.*, 2009). The Δ*covR* mutant showed a thicker capsule and longer chains. This was in agreement with the finding that *cps*2C and the gene encoding CMP-N-acetylneuraminic acid synthetase were among the upregulated genes in the Δ*covR* mutant. The gene *cps*2C has been proposed to be involved in chain length determination and export of polysaccharides for capsule production (Smith *et al.*, 1999).

The capsule of *S. suis* is poorly immunogenic. Sera from convalescent or bacterin immunized piglets generally have very low specific antibody titers directed against the capsular polysaccharides (Campo Sepulveda *et al.*, 1996; Wisselink *et al.*, 2001; Baums *et al.*, 2009). Immunization of piglets with polysaccharides elicited opsonizing antibodies only in the presence of Feunds' incomplete adjuvant (Elliott *et al.*, 1980). We induced very high capsule-specific serum IgG titers through immunization with conjugated serotype 2 capsular polysaccharides. The respective sera caused opsonization of the homologous serotype 2, but not the heterologous serotype 9 strain, and subsequent killing by porcine neutrophils (Baums *et al.*, 2009). Therefore, we think that a combination of conjugated polysaccharides from different serotypes might elicit protection in swine against *S. suis*, similar to immunoprophylaxis of pneumococcal infections in humans.

Peptidoglycan

In the cell wall of Gram-positive bacteria glycan strands consisting of alternating β-1,4-linked N-acetylglucosamine and N-acetylmuramic acid are cross-linked by short peptide chains (Vollmer *et al.*, 2008). Fittipaldi *et al.* (2008a) investigated muropeptides of *S. suis* using two different experimental approaches. The muropeptides had mainly D-isoglutamine at position 2 and L-lysine at position 3 of the peptide stem. In contrast to most other Gram-positive bacteria, *S. suis* peptidoglycan lacks interpeptide bridges but contains direct 3–4 cross-links. By high-sensitive Fourier transform mass spectrometry, the authors were able to detect N-deacetylated muropeptides in very low quantities in *S. suis* (probably below 1% of the total muropeptides). N-deacetylated peptidoglycan was not detectable in an *S. suis* Δ*pgd*A mutant confirming the proposed function of the protein encoded by the *pgd*A gene, which was originally identified as a gene upregulated upon interaction with porcine BMECs (Fittipaldi *et al.*, 2007). The peptidoglycan polysaccharide deacetylase PgdA from *S. suis* shares homology to PgdA from *Streptococcus pneumoniae*, in particular with respect to catalytic residues, and has also a predicted N-terminal membrane anchor. In general, modification of peptidoglycan through deacetylation results in lysozyme resistance (Vollmer, 2008). *S. suis* is very sensitive to lysozyme *in vitro*, most likely related to the low content of N-deacetylated muropeptides. Furthermore, inactivation of the *pgd*A gene does not result in increased lysozyme susceptibility. However, these phenotypes might be different *in vivo*, as *pgd*A is substantially upregulated upon interaction with host cells and within the host (Fittipaldi *et al.*, 2008a).

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The *S. suis pgd*A mutant was attenuated in virulence in murine and porcine models of infection. The impairment in virulence was associated with an accelerated decrease in numbers of bacteria in the blood. In accordance, the *pgd*A mutant was killed efficiently by neutrophils in the presence of naïve serum *in vitro*, in contrast to the wild-type strain. Furthermore, cytokine production, in particular IFN-γ and IL-6, was severely diminished in CD1 mice infected with the *pgd*A mutant in comparison to wild-type infected animals (Fittipaldi *et al.*, 2008a).

Li *et al.* (2009) performed selective capture of transcribed sequences (SCOTS) to identify genes preferentially expressed under iron starvation. At least six of the 63 identified upregulated genes encode proteins homologous to enzymes involved in cell wall biosynthesis. The upstream sequences of the genes *mur*G (SSU05_0477) and *mur*A (SSU05_1170) contain putative Fur boxes, which suggests that these genes are regulated by the iron-dependent transcriptional regulator Fur (Li *et al.*, 2009), and, thus, might be regulated during infection.

Cell wall components of *S. suis* exacerbate the host inflammatory response to infection as they induce a significant release of proinflammatory cytokines (TNF-α, IL-1β and IL6) and chemokines (IL-8 and MCP-1) (Segura *et al.*, 2006). Production of these molecules, in particular IL-6, IL-8 and MCP-1, is induced not only in inflammatory cells but also in BMEC (Vadeboncoeur *et al.*, 2003). This induction appears to be cell type specific as it does not occur in human umbilical vein endothelial cells. Production of chemokines by BMECs might be crucial for the inflammatory reaction induced by *S. suis* invasion of brain tissue and an important source of cytokines and chemokines in the cerebrospinal fluid.

Teichoic and lipoteichoic acids

Teichoic acid (TA) is a secondary polymer of α-glycerol phosphate. Lipoteichoic acid (LTA) contains a glycolipid moiety, resulting in an amphiphilic molecule. Elliot *et al.* (1977) proposed that LTA from *S. suis* has a backbone structure similar to TA from *Streptococcus pyogenes*, but with differences in glucosyl substituents. LTA and TA of *S. suis* were characterized earlier as antigens recognized by group D antiserum (Elliott *et al.*, 1977). Equivocal reactions described in Lancefield typing of *S. suis*, in particular classification as groups R, S and T, were mainly due to extraction of antigens from capsular material rather than from the cell wall as described by Gottschalk *et al.* (2007) in more detail.

TA and LTA might contribute to the adherence of pathogenic streptococci and staphylococci to host cells. In *S. suis*, the adhesion to porcine BMEC was reduced by pretreatment of the cells with LTA suggesting that LTA is partially involved in the adhesive phenotype of *S. suis* to these cells, which form part of the BBB (Vanier *et al.*, 2007). Upon interaction with porcine BMECs *S. suis* upregulates expression of DltA, an enzyme that catalyzes the D-alanylation of LTA (Fittipaldi *et al.*, 2007, 2008b). This modification of LTA is crucial for resistance against cationic antimicrobial peptides, most likely through reduction of the global negative charge of the bacterial envelope. Accordingly, a Δ*dlt*A mutant strain, but not the wild-type, was efficiently killed by porcine neutrophils in the presence of naïve serum and was attenuated in virulence in the CD1 mouse model (Fittipaldi *et al.*, 2008b).

Pili

Pilus formation has been detected in a number of Gram-positive bacteria including the streptococcal species *S. pyogenes*, *S. agalactiae* and *S. pneumoniae* (Telford *et al.*, 2006). In these streptococci, the genes encoding pili are located on pathogenicity islands. In *S. pneumoniae*, pili have been demonstrated to be important for adhesion to lung epithelial cells and for virulence in an intranasal challenge experiment with mice (Barocchi *et al.*, 2006). Ultrastructural investigations of *S. suis* revealed surface appendages with a length of approximately 250 nm and a width of 2 nm resembling pili of other Gram-positive pathogens (Jacques *et al.*, 1990). Accordingly, sequence analysis of the two serotype 2 reference strains P1/7 and 89-1591 indicated presence of a truncated homologue of the *S. agalactiae* pilus island 2b (PI-2b), which included the two genes encoding the ancillary and major pilin subunits (named *srt*F cluster by Takamatsu *et al.*, 2009). Importantly, the sequence of the putative major pilin subunit includes an E-box motif, which has been identified to be crucial for pilus assembly in other bacteria (Ton-That *et al.*, 2004; Telford *et al.*, 2006). The first gene (*ssu*0424 or *sip*F) of the series of genes that encode the truncated version of PI-2b in *S. suis* encodes a putative signal peptidase that is preferentially expressed by *S. suis* upon interaction with porcine BMECs (Fittipaldi *et al.*, 2007). Several mutations are present in the pilus gene cluster of *S. suis* strains P1/7 and NCTC 10237. Therefore, these strains might not express functional pilus structures. In the future, it is important to clarify whether the gene products of the truncated version of PI-2b in *S. suis* lead to pilus formation and whether this pilus is important for interaction with host cells and pathogenesis of *S. suis*.

Takamatsu *et al.* (2009) described a putative pilus gene cluster *srt*BCD with structural homology to the *rlr*A island of pneumococci. The cluster lacks a gene encoding a protein homologous to RlrA, but includes genes encoding putative pilin subunit proteins with E-box and LPXTG motifs and three class C sortases.

Sortases

Sortases are transpeptidases that anchor secreted proteins covalently to the cell wall with a C-terminal cell wall

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Table 1. Confirmed (c) and proposed (p) *S. suis* proteins with a CWS

Protein[a]	Theoretical MW (kDa)[b]	Confirmed (c) or proposed (p) function	References
SSU98_0465; SSU0473 (p)	180	Ribonuclease G and E (p)	(Chen *et al.*, 2007)
SSU05_2103; SSU98_2107; Lpx279 (Lpx31) (p)	150	Unknown	(Chen *et al.*, 2007; Jiang *et al.*, 2009)
MRP (c) (SSU0706)	136	Unknown	(Smith *et al.*, 1992)
SSU98_0812; SSU05_0812 (p)	136 (172)	C5a peptidase (p)	(Chen *et al.*, 2007)
HylA (c)	130	HylA (c)	(Allen *et al.*, 2004; King *et al.*, 2004)
SsnA (c)	108	DNA nuclease (c)	(Fontaine *et al.*, 2004; Zhang and Lu, 2007)
Sfp2 (p) (SSU0426)	108	Ancillary pilus subunit (p)[c]	(Fittipaldi *et al.*, 2007; Takamatsu *et al.*, 2009)
OFS (c)	105	Serum opacification (c)	(Baums *et al.*, 2006)
SntA (c) (SSU98_2098; SSU05_2095)	85	Cyclo-nucleotide-phosphodiesterase (p)	(Osaki *et al.*, 2002; Li *et al.*, 2009)
SSU98_01675 (c)	81	Unknown	(Geng *et al.*, 2008)
SSU0860 (c)	77	5′-nucleotidase (p)	(Geng *et al.*, 2008)
SSU98_0267 (c)	77	Translation initiation factor 2 (p)	(Geng *et al.*, 2008)
SAO (c)	75	Unknown	(Li *et al.*, 2006)
SntC (p) (SSU98_1013; SSU05_1000)	74	5′-nucleotidase (p)	(Osaki *et al.*, 2002)
SntB (p) (SSU98_1549 (c), SSU05_1538)	70	5′-nucleotidase (p)	(Osaki *et al.*, 2002; Geng *et al.*, 2008)
SIP1 (c) (SSU98_0197)	61	Unknown	(Geng *et al.*, 2008)
Sfp1 (p) (SSU05_0474; SSU98_0466)	51	Main pilus subunit (p), autotransporter adhesion (p)[c]	(Chen *et al.*, 2007; Fittipaldi *et al.*, 2007; Takamatsu *et al.*, 2009)
SSU1889	51	Porcine BMEC adhesin and invasin (p)	(Vanier *et al.*, 2009)
ORF203 (p)	31	Unknown	(Osaki *et al.*, 2002)
SSU05_0179 (p)	19	RTX family exoprotein A (p)	(Chen *et al.*, 2007; Liu *et al.*, 2009)

[a]The prefixes SSU98 and SSU05 in the ordered locus names have been annotated in the genomes of the two sequenced STSS strains 98HAH12 and O5ZYH33, respectively. Factors are regarded as proposed, if no confirmation of protein expression has been reported.
[b]The theoretical molecular weight is based on the genome sequence of serotype 2 reference strain P1/7 (www.sanger.ac.uk), if it is not provided by the authors.
[c]Further genes and pseudogenes encoding putative pilin subunit proteins with CWS have been described (see Table S2 in Takamatsu *et al.*, 2009, and Table 3 in Wang *et al.*, 2009).

sorting signal (CWS). The CWS includes an LPXTG- or related motif followed by a hydrophobic domain and a positively charged tail consisting of at least one arginine or lysine residue. For protein A of *Staphylococcus aureus*, it has been demonstrated that sortase A specifically cleaves the precursor protein between the threonine and the glycine residues and catalyzes the transfer of the processed protein to the free amino group of a penta-glycine cross-bridge of peptidoglycan (Mazmanian *et al.*, 1999; Ton-That *et al.*, 1999). *S. suis* serotype 2 carries five to six genes (*srt*A–F) encoding putative sortases, which is exceptional for streptococci (Osaki *et al.*, 2002; Wang *et al.*, 2009). The gene *srt*A was detected in *S. suis* strains of 28 different serotypes indicating that the gene is present in most if not all *S. suis* strains. It shows substantial allelic variation, which has been proposed to be a result of evolutionary divergence rather than recombination (Osaki *et al.*, 2003). The *S. suis* serotype 2 *srt*A gene displays high homology (65% identity) to the *srt*A of *Streptococcus gordonii*. The homology of the other *srt* genes (*srt*B–E) to known sortase genes is much lower. Furthermore, the putative *S. suis* sortases B–E are class C sortases that have a C-terminal hydrophobic domain serving most likely as a membrane anchor (Dramsi *et al.*, 2005). Osaki *et al.* (2002) found that more than 15 protein spots in 2D-PAGE of cell wall proteins depended on the presence of the gene encoding sortase A but were independent of other *srt* genes. Four putative sortase A substrates with a CWS were identified; among these was the muramidase-released protein (MRP, see also Table 1). Furthermore, the CWS-protein surface antigen one (SAO) was also not detectable on the surface of an *srt*A mutant (Wang *et al.*, 2009).

Isogenic *srt*A serotype 2 mutants are attenuated in adherence to and invasion of host cells including porcine BMECs, indicating that CWS proteins are involved in this interaction (Vanier *et al.*, 2008; Wang *et al.*, 2009). Accordingly, binding to plasma and cellular fibronectin

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as well as to collagen type I diminished in the *srt*A mutant. However, the binding of these host proteins is not completely abrogated in the *srt*A mutant, suggesting that the other sortases might also be involved in anchoring of adhesins or that non-CWS adhesins are also important for this phenotype (Vanier *et al.*, 2008).

Surprisingly, the isogenic *srt*A serotype 2 mutant was as virulent as the wild-type in the CD1 mice model (Vanier *et al.*, 2008). In contrast, intravenous infection of piglets with an *srt*A mutant of the Chinese STSS reference strain O5ZYH33 suggested attenuation in virulence. Competitive challenge experiments with the wild-type strain suggested that the *srt*A mutant is primarily attenuated in colonization of the brain and the lung (Wang *et al.*, 2009). Because of the contradictory findings, it is difficult at present to assess the relevance of sortase A function in the pathogenesis of *S. suis*.

Proteins with a CWS (LPXTG-motif)

In silico analysis of the genome of the Chinese serotype 2 reference strain O5ZYH33 revealed 33 putative cell wall-anchored proteins containing an LPXTG- or related motif (Wang *et al.*, 2009). As described above, LPXTG-motifs, in particular CWS, indicate covalent linkage of these proteins to the cell wall. In other streptococci, numerous LPXTG-proteins have been demonstrated to be involved in binding of extracellular matrix proteins and adhesion to host cells. Though it is accepted that *S. suis* interacts with extracellular host proteins and cells (Esgleas *et al.*, 2005), conclusive evidence for such a function is lacking for any of the putative adhesins described in *S. suis*. Following, surface-associated factors of *S. suis* are described that contain a CWS (see also Table 1).

MRP

Differences in virulence between *S. suis* serotype 2 strains are associated with expression of MRP and extracellular factor (EF). However, MRP is not an essential virulence factor for *S. suis* serotype 2, as an isogenic *mrp* mutant was as virulent as the wild-type strain (Smith *et al.*, 1996). The 136 kDa MRP was discovered as a factor released from virulent serotype 2 strains after muramidase treatment. The protein is also detectable in stationary-growth-phase culture supernatants of MRP expressing serotype 2 strains (Vecht *et al.*, 1991). Sequence analysis revealed a signal sequence and a CWS (Smith *et al.*, 1992). Accordingly, MRP has been demonstrated to be a substrate of sortase A (Osaki *et al.*, 2002). The mature MRP contains at the C-terminus a proline rich region followed by three repeats. The function of MRP is unknown. Fibronectin binding was not detectable (Smith *et al.*, 1992).

When compared to the early exponential growth phase *in vitro* expression of MRP is induced during infection (Tan *et al.*, 2008b). Accordingly, we demonstrated *in situ* MRP expression in fibrinosuppurative meningitis and granulomatous encephalitis lesions caused by experimental *S. suis* infections (Beineke *et al.*, 2008).

Expression of the 136 kDa MRP was found among strains of serotypes 1, 2, 1/2, 14 and 15. Large (MRP*) and small (MRP^{s}) size variants have been described, which were detectable in *S. suis* strains of nearly all serotypes investigated (Wisselink *et al.*, 2000). Serotype 1 MRP^{s} EF+ strains caused high mortality in germfree piglets within 48 h after intranasal infection at a dose as low as 10^4 CFU. Noteworthy, an isogenic *mrp*^{s} mutant of such a serotype 1 strain was not attenuated in virulence (Smith *et al.*, 1996). In central Europe, approximately 20% of the invasive *S. suis* diseases are caused by MRP* EF− serotype 9 strains (Wisselink *et al.*, 2000; Silva *et al.*, 2006). Duplication of intermediate and repetitive sequences present in *mrp* coding for the 136 kDa protein is responsible for the larger size of MRP* in these serotype 9 strains (Silva *et al.*, 2006). In serotype 7 strains, size variation of *mrp* is very pronounced. However, Western blot analysis suggested that most of these *mrp* variant genes in serotype 7 strains are silent (Silva *et al.*, 2006).

Determination of the *mrp* and *epf* genotypes (or the respective phenotypes) in addition to serotyping has been performed in numerous epidemiological studies (Gottschalk *et al.*, 1998; Wisselink *et al.*, 2000; Silva *et al.*, 2006; Wei *et al.*, 2009). It is now included in routine diagnosis of *S. suis* in several laboratories. This differentiation allows identification of the two most important European *S. suis* pathotypes, *mrp*+ *epf*+ *cps*2 (MRP+ EF+ serotype 2) and *mrp** *epf*− *cps*9 (MRP* EF− serotype 9) strains. However, due to some open questions interpretation of typing results remains difficult. Though *mrp* (MRP) is a virulence marker in serotype 2 strains, it is not clear whether this is also the case for other serotypes. Furthermore, MRP+ EF− serotype 2 strains have not been compared to other serotype 2 phenotypes (MRP+ EF+, MRP+ EF* and MRP− EF−) in experimental infections with piglets, which makes it difficult to assess the virulence of this particular phenotype. Recent experimental infections of BALB/c mice suggested that *mrp*+ *epf*− *cps*2 strains are substantially less virulent than *mrp*+ *epf*+ *cps*2 genotype strains (Wei *et al.*, 2009). However, determination of the virulence of an *S. suis* strain in mice is problematic, since results obtained from BALB/c mice and piglet infections differ from each other (Vecht *et al.*, 1997). As *mrp* seems to be a silent gene in some strains (e.g. serotype 7), it might also be discussed that phenotyping rather than genotyping should be performed in diagnostic laboratories. Nevertheless, for the identification of the two most important European *S. suis* pathotypes, *mrp*+ *epf*+ *cps*2 and *mrp** *epf*− *cps*9, as well as the highly virulent *mrp*^{s} *epf*+ *cps*1 pathotype, both approaches appear equally reliable (Wisselink *et al.*, 2002; Silva *et al.*, 2006). Importantly, neither genotyping nor phenotyping of these markers alone allows classification of a strain as avirulent, as MRP and EF are not crucial

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virulence factors (Smith *et al.*, 1996), and MRP− EF− serotype 2 strains from North America induce disease in experimental infections (Gottschalk *et al.*, 1999).

MRP is a highly immunogenic protein. Convalescent sera from piglets infected with either MRP+ serotype 2 or MRP* serotype 9 strains generally contain high specific antibody titers (Zhang and Lu, 2007). Furthermore, immunizations with bacterins based on such strains elicited seroconversion against MRP (Wisselink *et al.*, 2001; Baums *et al.*, 2009).

Geng *et al.* (2008) observed 90% bactericidal activity against *S. suis* serotype 2 in a whole human blood killing assay with anti-rMRP antisera. The bactericidal activity of sera against 10 other *S. suis* cell wall-associated proteins was much lower. In contrast, we did not observe elicitation of opsonizing antibodies in piglets by immunization with a subunit vaccine including MRP, though these animals developed high antibody titers against MRP (Baums *et al.*, 2009). The lack of opsonizing activity in post immune sera of this group correlated with the lack of protection in serotype 2 and 9 challenges. Accordingly, Wisselink *et al.* (2001) also found no protection in MRP+ EF+ serotype 2 challenge experiments of piglets after application of a vaccine based on purified MRP, in contrast to immunization with a bacterin and a vaccine containing MRP and EF (Table 4). In conclusion, at present it seems more likely that MRP specific antibodies in swine are not protective, though they are good markers of infection.

Hyaluronidase (HylA)

Hyaluronic acid (HA) is a glycosaminoglycan that constitutes a major part of the extracellular matrix and the capsule of certain bacteria. The majority of *S. suis* strains of serotypes 3 and 7 degrade HA by secreting a 130 kDa hyaluronidase (also referred to as hyaluronate lyase) (Allen *et al.*, 2004; King *et al.*, 2004). Furthermore, cultivation experiments indicated that these strains ferment the degradation product of HA. In contrast hyaluronidase activity is not detectable in most strains of other serotypes including 1, 1/2, 5, 6, 9, 10, 14 and 22. Some virulent serotype 2 reference strains such as P1/7, and strains of serotypes 1, 1/2, 5 and 14, express truncated versions of the protein, which is not associated with hyaluronidase activity. Sequence analysis of the full length 130 kDa hyaluronidase predicts a CWS (in contrast to the C-terminal truncated versions) (King *et al.*, 2004). However, enzyme activity is detectable in the culture supernatant (Allen *et al.*, 2004). This is in agreement with the presence of MRP and CWS proteins from other bacteria in culture supernatants (Vecht *et al.*, 1991; Courtney *et al.*, 1999).

The expression pattern of hyaluronidase among different *S. suis* strains indicates that hyaluronidase activity is not crucial for the pathogenesis of *S. suis* meningitis and other invasive diseases, as highly virulent *S. suis* strains lack this activity. On the other hand, it cannot be excluded that expression of hyaluronidase is important for the pathogenesis of diseases (e.g. pneumonia) associated with different pathotypes, in particular serotype 7 (King *et al.*, 2004). Noteworthy, high hyaluronidase activity was observed in strains of serotype 7. Interestingly, Allen *et al.* (2004) observed an inverse correlation between the presence of suilysin and hyaluronidase activity. Furthermore, genetic analysis of *hyl*A revealed high diversity in the 5′-end and evidence that recombination has contributed to molecular variation. Some *hyl* alleles possess direct sequence duplications of 2 or 4 bp, which are known to be intrinsically unstable. It is plausible to speculate that expression of hyaluronidase is regulated by the generation and excision of these repeats during infection (King *et al.*, 2004).

SAO

Li *et al.* (2006) discovered SAO as an immunogenic protein of *S. suis* which is recognized by porcine convalescent sera (Table 1). The authors proved surface localization by immuno gold-labeling electron microscopy. The protein contains a CWS and numerous repeats in its C-terminal half. Variations in the number of repeats account for differences in molecular weight observed between SAO variants of different serotypes and within serotype 2 strains (Li *et al.*, 2006; Feng *et al.*, 2007). The function of SAO is still unknown.

Immunization of mice and piglets with rSAO elicited protective immune response against serotype 2 strains expressing different variants of SAO (Li *et al.*, 2007). Furthermore, Zhang *et al.* (2009) described protection against challenges with serotypes 2 and 7 strains in mice after immunization with rSAO. Protection in piglets was observed when using the adjuvant Quil A (Brenntag Biosector, Frederikssund, Denmark), but not with Emulsigen-Plus (MVP Laboratories, Ralston, USA) (Li *et al.*, 2006, 2007). In contrast to piglets immunized with SAO and Emulsigen-Plus, application of Quil A elicited an IgG2 dominated immune response. The authors discussed correlation of protection with a putative Th-1 response and induction of opsonizing antibodies. However, we demonstrated that, in contrast to a serotype 2 bacterin, a subunit vaccine including SAO did not elicit opsonizing antibodies or protection against serotype 2 or 9 challenges, despite high SAO specific IgG2 titers (Baums *et al.*, 2009). The different results on protective properties of SAO might be explained by different experimental conditions used to test vaccine efficacy (Table 4).

Opacity-factor of Streptococcus suis *(OFS)*

S. suis serotype 2 (multilocus sequence type 1) expresses a surface-associated protein called OFS, which opacifies serum of various species (Baums *et al.*, 2006, see also Table 1). The protein has structural features of an MSCRAMM, namely a signal sequence, a large N-terminal domain, C-terminal repeats and a CWS. The N-terminal domain is responsible for serum opacification and is homologous to FnBA of *Streptococcus dysgalactiae* and serum opacity factor (SOF) of *S. pyogenes*. In contrast

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to these MSCRAMMs, OFS does not confer binding to fibronectin. This might be explained by substantial sequence deviation found in the repeats of OFS in comparison to the repeats of other MSCRAMMs. Takamatsu *et al.* (2008) identified three additional genotypes of *ofs*. In contrast to the originally described OFS (designated type-1 OFS by Takamatsu *et al.*, 2008), which possesses three C-terminal repetitive sequences, the type-2 OFS is characterized by four repetitive sequences. In strains expressing type-2 OFS, serum opacification activity was detectable in culture supernatants. In contrast, type-1 OFS activity was only detected in SDS-extracts of bacteria. In addition to type-1 and type-2 *ofs*, other variants of *ofs* were identified which contain either point mutations or genetic disruptions by insertion elements or rearrangements. Accordingly, these did not express detectable serum opacification activity (Takamatsu *et al.*, 2008).

Using an isogenic *ofs* in-frame deletion mutant, we demonstrated that OFS is an important virulence factor in *S. suis* serotype 2 (multilocus sequence type 1). Though few animals developed disease after intranasal infection with the *ofs* mutant, the majority of piglets did not show any sign of disease, in contrast to the wild-type strain that caused mortalities of 70–90% (Baums *et al.*, 2006; Kock *et al.*, 2009). However, the function of OFS in pathogenesis might be restricted to the highly virulent multilocus sequence type (ST) complex 1 as serum opacification activity is not detectable in virulent strains of ST complex 27 (Takamatsu *et al.*, 2008).

Interestingly, the *ofs* mutant was not attenuated in colonizing the upper respiratory tract and the tonsils. Therefore, we tested the *ofs* mutant as a live vaccine candidate for intranasal application. The mortality after homologous challenge was lower in the vaccinated piglets than in the control animals; however, this difference was not significant. Furthermore, the serotype 2 *ofs* mutant vaccine did not elicit protection against the heterologous serotype 9 challenge (Kock *et al.*, 2009).

Immunization experiments with rOFS demonstrated that the protein is immunogenic (our unpublished results). On the other hand, we could not detect antibody titers against OFS in convalescent sera, suggesting that this protein is expressed only in limited amounts and only during a short period of infection. The possible role of OFS in *S. suis*–host interactions remains to be elucidated.

Putative cyclic nucleotide phosphodiesterase (SntA)

S. suis sortase A anchors SntA, a putative 2′,3′-cyclic nucleotide 2′-phosphodiesterase, to the cell wall (Osaki *et al.*, 2002). The expression of *snt*A (also called *cpd*B) is upregulated under iron starvation (Li *et al.*, 2009). To the best of our knowledge the function of SntA in *S. suis* has yet not been identified. As SntA possesses an RGD motif, it is plausible to speculate that SntA might be involved in interaction with host cells by binding to integrin(s) on their surface (Osaki *et al.*, 2002).

Proposed and confirmed surface-associated proteins lacking a known CWS

Fibronectin- and fibrinogen-binding protein of S. suis *(FBPS)*

The gene encoding FBPS was identified by *in vivo* promoter selection (Smith *et al.*, 2001). Accordingly, Tan *et al.* (2008b) observed higher expression of *fbps in vivo* than in early exponential growth phase cultures. Sequence comparisons revealed homology of FBPS with the fibronectin binding proteins FlpA and FBP54 of *S. gordonii* and *S. pyogenes*, respectively. This homology is very prominent (80%) in the fibrionogen/fibronectin binding region of FBP54. Accordingly, rFBPS binds to human fibronectin and fibrinogen (de Greeff *et al.*, 2002, see also Table 2). *S. suis* serotype 2 binds to both plasma and cellular fibronectin (Esgleas *et al.*, 2005). However, so far it has not been demonstrated that FBPS confers binding of *S. suis* to fibronectin (or fibrinogen).

The *fbps* serotype 2 knock-out mutant was tested in a competition challenge experiment with the wild-type strain in four piglets (de Greeff *et al.*, 2002). The results suggested that the *fbps* mutant is attenuated in virulence but not in colonization of the tonsils.

The *fbps* gene is widely distributed among different serotypes of *S. suis*. It is detectable in virulent, weakly virulent and avirulent serotype 2 strains (de Greeff *et al.*, 2002). Allelic variation of *fbps* has not been described. Whether FBPS is expressed in all *fbps*-positive strains is also unknown.

Convalescent-phase sera recognize FBPS. The protective efficacy of a vaccine based on rFBPS or purified FBPS in pigs has not been reported. Immunization of BALB/c mice with rFBPS did not elicit significant protection against intra-peritoneal challenge with the Chinese STSS reference strain O5ZYH33, in contrast to immunization with other *S. suis* antigens (Liu *et al.*, 2009). A subunit vaccine including FBPS and other surface-associated proteins was also not protective in serotypes 2 and 9 challenge experiments of piglets (Baums *et al.*, 2009, see also Table 4).

Arginine deiminase (ArcA)

Arginine deiminase (ArcA) belongs to the Arginine–Deiminase system (ADS), a catabolic enzymatic pathway known in many bacteria, including streptococci. ADS catalyzes the conversion of arginine to ornithine, carbon dioxide and ammonia, thereby generating ATP (Cunin *et al.*, 1986; Zuniga *et al.*, 2002).

Some years ago we identified an ADS in *S. suis* as a temperature-induced and partially cell wall associated enzyme system (Winterhoff *et al.*, 2002, see also Table 2). It is encoded by three major genes, *arc*A, *arc*B and *arc*C, which are transcribed from an operon (AD operon). *arc*A codes for an arginine deiminase (AD), *arc*B for an ornithine-carbamoyltransferase (OCT) and *arc*C for a

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Table 2. Confirmed (c) and proposed (p) surface-associated proteins of *S. suis* without a CWS[a]

Protein[b]	Theoretical MW (kDa)	Confirmed (c) or proposed (p) function	References
Neprilysin[c] (c)	67	Zn^{2+} metalloprotease[c] (p)	(Wu *et al.*, 2008b)
SSU1664 (c)	66	Oligopeptide-binding protein OppA (p)	(Geng *et al.*, 2008)
FBPS (c)	54	Fibronectin- and fibrinogen-binding protein (c)	(de Greeff *et al.*, 2002)
SSU05_0544[c] (c)	54	Pyruvate kinase[c] (p)	(Chen *et al.*, 2007; Wu *et al.*, 2008b)
PdgA (c) (SSU1448)	52	Peptidoglycan polysaccharide deacetylase (c)	(Fittipaldi *et al.*, 2007, 2008b)
6PGD (c)	52	6-phosphogluconate-dehydrogenase, adhesin (p)	(Tan *et al.*, 2008a)
DltB (SSU0597)	49	Membrane bound O-acyl transferase (p)	(Fittipaldi *et al.*, 2007)
SSU05_860[c] (c)	48	Peptide ABC transporter peptide-binding protein[c] (p)	(Chen *et al.*, 2007; Wu *et al.*, 2008b)
Eno (c) (SSU1320)	47	Enolase, plasminogen binding (c)	(Esgleas *et al.*, 2008)
Esb[c] (c)	47	Extracellular solute-binding protein[c] (p)	(Wu *et al.*, 2008b)
ArcA (c)	46	Arginine deiminase (c)	(Winterhoff *et al.*, 2002; Gruening *et al.*, 2006)
38 kDa antigen (c)	46	Unknown	(Okwumabua and Chinnapapakkagari, 2005)
DltA (SSU1184) (c)	39	D-alanine–D-alanine ligase (c)	(Fittipaldi *et al.*, 2007, 2008b)
GAPDH (c)	39	Glyceraldehyde-3-phosphate dehydrogenase, adhesin, plasminogen binding (c)	(Brassard *et al.*, 2001, 2004; Jobin *et al.*, 2004)
Ef-Ts[c] (c)	37	Translation elongation factor[c] (p)	(Wu *et al.*, 2008b)
(c)	34	Phosphate acetyltransferase[c] (p)	(Wu *et al.*, 2008b)
LBP (SSU05_0330) (p)	34	Laminin-binding protein (p)	(Pan *et al.*, 2009)
FBA[b] (SSU98_0330) (c)	31	Fructose-biphosphate aldolase[c] (p)	(Wu *et al.*, 2008b)
SSU1487 (p)	20	VanZ-like protein (p)	(Fittipaldi *et al.*, 2007)

[a]Integral membrane proteins are not included with the exception of a few selected proteins with a C-terminal membrane anchor.
[b]The prefixes SSU98 and SSU05 in the ordered locus names have been annotated in the genomes of the two sequenced STSS strains 98HAH12 and O5ZYH33, respectively. Factors are regarded as proposed, if no confirmation of protein expression is available.
[c]These factors were identified as surface-associated factors in *S. suis* serotype 9 based on an immunoproteomic analysis of cell wall proteins (Wu *et al.*, 2008a).

carbamate kinase (CK) (Gruening *et al.*, 2006). The AD operon is clustered together with a putative arginine–ornithine antiporter (*arc*D), a putative Xaa-His dipeptidase (*arc*T) and a putative endo-beta-galactosidase (*arc*H). The operon seems to be controlled by several factors, including carbon catabolite repression by CcpA, metabolic regulation by an fumarate and nitrate reduction regulator (FNR)-like protein (FlpS) and by the ArgR repressor belonging to the ArgR/AhrC family (Gruening *et al.*, 2006).

A putative role of the ADS during streptococcal pathogenesis was first reported for *S. pyogenes*, since it was shown to be involved in epithelial cell adhesion and invasion of this pathogen (Degnan *et al.*, 2000). In *S. suis*, we have recently found that expression of the ADS favors survival of *S. suis* in acidified epithelial cell compartments. Furthermore, we observed that mutants defective in different structural and regulators genes of the ADS were defective in production of ammonia and neutralization of low pH during *in vitro* growth, as well as in survival at low pH, low oxygen and inside HEp-2 epithelial cells (Benga *et al.*, 2004; Gruening *et al.*, 2006). The relevance of the ADS for pathogenesis remains to be elucidated. Nevertheless, expression of ArcA on the streptococcal surface, and the phenotype of AD mutants with respect to biological fitness in *in vitro* culture and host epithelial cells suggest a role of the ADS in *S. suis* survival during infection.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH is an enzyme of the glycolytic pathway and, in addition, a surface-associated factor in pathogenic streptococci involved in interaction with host proteins and cells (Pancholi and Fischetti, 1992). Surface-associated GAPDH was discovered in *S. suis* as an albumin-binding protein (Quessy *et al.*, 1997). Cloning and sequencing of *gapdh* from *S. suis* serotype 2 revealed high homology to the homologous genes of other streptococci, including *S. pneumoniae* and *S. pyogenes* (Brassard *et al.*, 2004, see also Table 2). Transposon mutants deficient in the expression of GAPDH were attenuated in the adhesion to embryonic bovine tracheal cells and porcine tracheal rings, suggesting that GAPDH is involved in adhesion to host cells (Brassard *et al.*, 2001). Furthermore, plasminogen binding to the surface of *S. suis* was reduced by approximately 25% in a *gapdh* transposon mutant,

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suggesting that *gapdh* is also involved in plasminogen acquisition (Jobin *et al.*, 2004). Plasminogen is a central component of the host fibrinolytic system. Host plasmin activators or bacterial proteases, such as streptokinase, may activate plasminogen generating the serine protease plasmin. Different pathogens exploit the fibrinolytic activity of plasmin(ogen) by binding and activating plasminogen on their surface. In *S. suis*, activation of surface-bound plasminogen does not occur in the presence of serum, but after addition of urokinase, streptokinase or supernatant from *S. dysgalactiae* subsp. *equisimilis* (Jobin *et al.*, 2004). Interestingly, co-incubation experiments with human BMEC indicated that these cells constitutively secrete plasminogen activators, which might activate plasminogen on the surface of *S. suis* (Jobin *et al.*, 2005a).

Enolase

Similar to GAPDH, enolase is not only a cytosolic enzyme of the glycolysis pathway but also a surface-associated factor of various pathogenic streptococci involved in their interactions with host proteins (Pancholi and Fischetti, 1998). One important function of streptococcal surface enolase (SEN) seems to be binding of plasmin(ogen). Activation of the fibrinolytic activity of host plasmin (ogen) on the bacterial surface is considered an important mechanism of tissue invasion. Esgleas *et al.* (2008) identified SEN of *S. suis* in search of a fibronectin-binding protein (Table 2). The authors confirmed that rSEN from *S. suis* has plasminogen- and fibronectin-binding activity with comparable high affinity. Noteworthy, SEN from *S. suis* is the only known SEN that binds to fibronectin. It is, however, unknown, whether *S. suis* fibronectin-binding properties depend on expression of SEN.

Enolase is an immunogenic protein as convalescent sera and sera from bacterin immunized piglets contain specific antibodies (Zhang and Lu, 2007). Immunization experiments of mice with recombinant enolase have elicited contradictory results. In BALB/c mice, significant protection against serotype 2 and 7 intra-peritoneal challenges was described using complete Freund's adjuvant (Zhang *et al.*, 2009). In contrast, no protection was observed in CD1 mice using QuilA as adjuvant (Esgleas *et al.* 2009, see also Table 4). As pathology in CD1, but not in BALB/c mice, includes meningitis, it remains questionable whether enolase immunization can protect against invasive *S. suis* infections.

Hemagglutinins

S. suis expresses a lectin-like adhesin that binds to the disaccharide Gal-(α1–4)-Gal of the glycolipid trihexosylceramide (GbO_3) (Haataja *et al.*, 1996). This binding activity causes hemagglutination and is associated with an 18 kDa protein, which is classified into subtypes P_N and P_O (Tikkanen *et al.*, 1995). N-acetylgalactosamine inhibits binding of P_N only, whereas galactose inhibits binding of P_N and P_O. The Gal-(α1–4)-Gal binding adhesin is present in strains of various serotypes (Tikkanen *et al.*, 1996). Encapsulation is thought to interfere with the accessibility of this adhesin. However, preliminary immunization experiments in mice with the purified Gal-(α1–4)-Gal adhesin indicated induction of opsonizing antibodies (Tikkanen *et al.*, 1996).

A different hemagglutinin expressed by only a limited number of strains binds to a sialylated poly-N-acetyllactosamine-containing component on the surface of erythrocytes (Liukkonen *et al.*, 1992). To the best of our knowledge, the genes encoding these hemagglutinins have not been identified.

Lipoproteins

Prolipoproteins contain a conserved sequence element in the signal sequence called 'lipobox', which leads to a specific processing pathway. A prolipoprotein diacylglyceryl transferase (Lgt) transfers a diacylglyceryl moiety from a membrane lipid onto the conserved lipobox cysteine forming a thioether linkage. Subsequently, a lipoprotein signal peptidase (Lsp) cleaves the prolipoprotein in the 'lipobox', leaving the lipid-modified cysteine at the N-terminus (Hutchings *et al.*, 2009). A putative *lgt* gene has been annotated in the genomes of the Chinese *S. suis* STSS strains O5ZYH33 (SSUO5_1605) and 98HAH33 (SSU98_1615), but so far it has not been characterized in further detail (Chen *et al.*, 2007). A highly homologous open reading frame (ORF) is also present in the genome of the European serotype 2 reference strain P1/7 (our unpublished results). An Lsp was identified by de Greeff *et al.* (2003). Sequence analysis of Lsp suggested four transmembrane spanning regions. An *lsp* knock out mutant of a virulent serotype 2 strain showed at least four band shifts of metabolically labeled lipoproteins indicating accumulation of some prolipoproteins in the *lsp* mutant. Accordingly, a band shift in this mutant in comparison to the wild-type strain was also observed in Western blot analysis with an antibody directed against the pneumococcal lipoprotein PsaA, a component of a Mn^{2+} transporter. In a competitive experimental infection experiment the *lsp* knock-out mutant was not attenuated in virulence as compared to the parental strain. These results are, however, difficult to interpret as some of the lipoproteins might be processed by an alternative, yet unidentified pathway as suggested by band shifts of the metabolically labeled lipoproteins in the respective mutant (de Greeff *et al.*, 2003).

In another recent study, three putative divalent-cation-binding lipoproteins of ABC transporters were identified and shown to be upregulated upon divalent-cation deprivation (Aranda *et al.*, 2008). Accordingly, the predicted regulator of Zn^{2+} and Mn^{2+} uptake AdcR was demonstrated to bind to two of the three promoters of the putative lipoprotein genes *in vitro*. Furthermore, in a mutant of a

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Table 3. Proposed (p) and confirmed (c) secreted factors of *S. suis* (selection)

Protein	Theoretical MW (kDa)	Confirmed (c) or proposed (p) function	References
ZmpC[a] (p) (SSU98_1036, SSU05_1022)	217	IgA1 protease (p)	(Chen *et al.*, 2007)
EF* (c)	>110	Unknown	(Smith *et al.*, 1993)
EF (c) (SSU0171)	110	Unknown	(Vecht *et al.*, 1991)
SSU1760 (c)	112	DNA nuclease (p)	(Zhang and Lu, 2007)
DPP IV[b] (c)	85	Dipeptidyl peptidase IV (c)	(Jobin *et al.*, 2005b)
SSU1215 (c)	74	Unknown	(Zhang and Lu, 2007)
SSU1664 (c)	66	Serine-type D-Ala–D-Ala carboxypeptidase (p)	(Zhang and Lu, 2007)
SSU0152 (c)	66	Unknown	(Zhang and Lu, 2007)
Truncated HylA (c)	58 (62, 70, 100)	Unknown	(Allen *et al.*, 2004)
Arg-aminopeptidase[b] (c)	55	Arg-aminopeptidase (c)	(Jobin and Grenier, 2003)
Suilysin (c)	54	Hemolysin (c), CDC	(Jacobs *et al.*, 1994)
IgG binding protein[c] (c)	52	IgG binding (c)	(Serhir *et al.*, 1995)
Hly (p) (SSU05_1668)	50	Hemolysin-like protein containing CBS domain (p)	(Chen *et al.*, 2007)
SSU0020 (c)	45	Unknown	(Zhang and Lu, 2007)
SerP (c) (SSU05_2192)	42	Serine protease (p)	(Chen *et al.*, 2007; Liu *et al.*, 2009)
SSU0457 (p)	32	Collagenase-peptidase (p)	(Osaki *et al.*, 2002; Fittipaldi *et al.*, 2007)
BLISs[d] (c)	14–30	Bacteriostatic (c)	(Melancon and Grenier, 2003)
SSU98_1819 (c)	12	Ribonuclease G and E (p)	(Geng *et al.*, 2008)
Unknown		Phospholipase C	(Jobin *et al.*, 2005a)

[a]This ORF was annotated as an LPXTG-motif cell wall anchor domain protein in SsuiDRAFT_3692 of strain 89/1591. However, the putative Gram-positive anchor identified by Pfam 5 is localized between amino acids 67 and 106 at the N-terminus.
[b]Cell-associated activity of DPP IV and Arg-aminopeptidase was also reported in the respective publications.
[c]An ORF encoding a putative IgG-binding protein is annotated in the genome of *S. suis* 98015 (accession no. DQ410872), but experimental evidence regarding IgG binding of this factor has not been published.
[d]Bacteriocin-like inhibitory substances.

different zinc uptake regulator (Zur), putative Zn^{2+}-binding lipoproteins were also found to be differentially regulated (Feng *et al.*, 2008). Interestingly, one of the three putative lipoproteins (SsuiDRAFT 0103) described by Aranda *et al.* (2008) induced significant protection in mice (Table 4). A second putative protective lipoprotein was identified in an immunoproteomic analysis of cell-wall associated proteins. The protein SSU98_1094 is predicted to be a lipoprotein of an uncharacterized ABC-type transport system (Geng *et al.*, 2008). Immunization of rats with rSSU98_1094 elicited high titers of opsonizing antibodies based on a whole human blood killing assay. Furthermore, convalescent sera from humans (meningitis or STSS patients) and piglets as well as from bacterin immunized piglets contain high serum antibody titers against SSU98_1094. Based on the results of this comprehensive immunoproteomic analysis of *S. suis* serotype 2, SSU98_1094 was suggested, together with two other surface-associated proteins (SSU98_0197 and SSU1664), as a vaccine candidate and diagnostic target (Geng *et al.*, 2008).

Secreted factors

Secreted proteins may have various functions in bacterial pathogenesis. For instance, depending on the concentration and/or tissue, toxins may either damage host cells or interfere with activation pathways thereby modulating host immune responses. An obvious difference from surface-associated factors is that activity of a secreted factor does not require bacteria–host cell contact, but depends on diffusion in tissue in order to reach critical concentration. In *S. suis*, a number of secreted proteins have been identified, which most likely represent only a small part of the secretome of this pathogen (Table 3).

Suilysin

Suilysin is a pore-forming cholesterol-dependent cytotoxin (CDC) expressed by many but not all virulent *S. suis* strains. It shares 52% amino acid identity with pneumolysin, its closest known relative within the CDC protein family (Segers *et al.*, 1998). In contrast to pneumolysin it contains a signal sequence and is secreted. The mature protein has an estimated molecular weight of 54 kDa. The hemolytic activity of suilysin is lost upon oxidation and is inhibited by cholesterol (Jacobs *et al.*, 1994). The susceptibility of erythrocytes to suilysin-mediated lysis shows some species specificity, with human group O erythrocytes being the most susceptible, followed by horse, sheep, cow and pig red blood cells (Gottschalk *et al.*, 1995).

Sequencing of *S. suis* genomes revealed additional genes encoding putative hemolysins (Chen *et al.*, 2007).

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Table 4. Evaluation of protective efficacies of *S. suis* proteins

Component(s)	Adjuvant	Animal model	Challenge serotype	Challenge Dose (CFU) and route	Protection	References
SLY	α-tocopherol	Mice	2	1×10^9 i.p.	Yes	(Jacobs *et al.*, 1994)
SLY	α-tocopherol	Piglets	2	1×10^9 i.p.	Yes?[a]	(Jacobs *et al.*, 1996)
MRP	Water-in-oil	SPF piglets	2	10^7 i.v.	No	(Wisselink *et al.*, 2001)
EF	Water-in-oil	SPF-piglets	2	10^7 i.v.	No	(Wisselink *et al.*, 2001)
MRP and EF	Water-in-oil	SPF piglets	2	10^7 i.v.	Yes	(Wisselink *et al.*, 2001)
38 kDa antigen	CFA, IFA[b]	Piglets	2	1.5×10^6 i.v.	Yes	(Okwumabua and Chinnapapakkagari, 2005)
r6PGD	CFA, IFA[b]	CD1 mice	2	2.5×10^8 i.p.	Yes	(Tan *et al.*, 2008a)
r6PGD	CFA, IFA[b]	SPF piglets	2	1×10^6 i.v.	Yes[c]	(Tan *et al.*, 2009)
rSsuiDRAFT 0103[d]	Imject Alumn	Balb/c mice	2	20 LD_{50s} i.p.	Yes	(Aranda *et al.*, 2008)
rSsuiDRAFT 0174[d]	Imject Alumn	Balb/c mice	2	20 LD_{50s} i.p.	No	(Aranda *et al.*, 2008)
rSsuiDRAFT 1237[d]	Imject Alumn	Balb/c mice	2	20 LD_{50s} i.p.	No	(Aranda *et al.*, 2008)
rSSU05_0179	CFA, IFA[b]	Balb/c mice	2	4×10^7 i.p.	Yes	(Liu *et al.*, 2009)[e]
rFBPS	CFA, IFA[b]	Balb/c mice	2	4×10^7 i.p.	No	(Liu *et al.*, 2009)[e]
rSAO	Emulsigen	SPF piglets	2	5×10^6 i.n.	No	(Li *et al.*, 2006)
rSAO	QuilA	SPF piglets	2	10^7 i.n.	Yes	(Li *et al.*, 2007)
rSAO	CFA, IFA[b]	Balb/c mice	2	2×10^9 i.p.	Yes	(Zhang *et al.*, 2009)
rSAO	CFA, IFA[b]	Balb/c mice	7	1×10^9 i.p.	Yes	(Zhang *et al.*, 2009)
rEnolase	CFA, IFA[b]	Balb/c mice	2	2×10^9 i.p.	Yes	(Zhang *et al.*, 2009)
rEnolase	CFA, IFA[b]	Balb/c mice	7	1×10^9 i.p.	Yes	(Zhang *et al.*, 2009)
rEnolase	QuilA	CD1 mice	2	1×10^8 i.p.	No	(Esgleas *et al.*, 2009)
MAP[f]-fraction	Emulsigen	SPF piglets	2	10^9 i.n.	No	(Baums *et al.*, 2009)
MAP[f]-fraction	Emulsigen	SPF piglets	9	10^8 i.v.	No	(Baums *et al.*, 2009)

[a]Immunization experiments with three piglets suggested protection.
[b]CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.
[c]Noteworthy, 50% mortality was observed in immunized piglets.
[d]Annotations in the genome of the North American reference strain 89/1591.
[e]The protective efficacies of eight other recombinant *S. suis* proteins were investigated in this study using the same protocol for immunization and challenge with the STSS strain 05ZYH33 (Liu *et al.*, 2009).
[f]Murein-associated protein.

However, our Blast searches with the published *S. suis* genome sequences did not reveal a further putative CDC in *S. suis* (unpublished). In this respect, Table 1 of the publication by Chen *et al.* (2007) listing *ply* and *sly* for *S. suis* is confusing. Apparently, both names refer to the suilysin gene (C. Chen, personal communication), and *S. suis* does not have a pneumolysin gene.

The *sly* gene displays very limited genetic diversity. Eighty percent of 208 investigated *sly*+*S. suis* isolates contained an identical *sly* allele as defined by restriction fragment length polymorphism (RFLP) analysis (King *et al.*, 2001). Based on sequencing and Southern blot analysis it was suggested that the *sly* gene was acquired from a foreign source and laterally transferred from strain to strain by a mechanism including natural transformation and homologous recombination (Takamatsu *et al.*, 2002).

In contrast to other virulence-associated genes such as *mrp* and *fbps*, expression of suilysin mRNA was found to be induced rather late during infection, i.e. at 48 h after intravenous application of the serotype 2 challenge strain (Tan *et al.*, 2008b). Noteworthy, manifestation of disease was observed at 20 h post infection. Depending on the tissue, a 2–4-fold induction of suilysin mRNA was described in comparison to *in vitro* expression. However, mRNA from *in vitro* cultures was collected at 3 h of cultivation. Therefore, it cannot be excluded that the postulated *in vivo* induction is simply due to the substantial upregulation of suilysin expression at the end of the exponential growth phase, as demonstrated by experiments measuring hemolytic activities (Gottschalk *et al.*, 1995) and *sly* promoter activity analysis (Lun and Willson, 2005). Interestingly, transposon interruption of the gene *man*N endocing mannose permease IID of the mannose-specific phosphotransferase system resulted in a hyper-hemolytic phenotype due to increased suilysin expression. As this phenotype was, at least partially, reverted by complementation with *man*N, it was concluded that regulation of *sly* can be mediated by the mannose-specific phosphotransferase system (Lun and Willson, 2005).

Suilysin has cytotoxic activity for various host cells, as shown for different epithelial cell lines (Norton *et al.*, 1999; Lalonde *et al.*, 2000), BMECs (Charland *et al.*, 2000) and macrophages (Segura and Gottschalk, 2002). As determined by lactate dehydrogenase activity, more than 80% cytotoxicity was seen using 1–10 μg/ml suilysin (Charland *et al.*, 2000; Lalonde *et al.*, 2000; Segura and Gottschalk, 2002). However, suilysin also induces other cellular responses at sublytic concentrations (see below).

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Invasion of epithelial cells by encapsulated serotype 2 strains is somewhat controversial, as already mentioned above. Norton *et al.* (1999) described invasion of HEp-2 epithelial cells by encapsulated *sly*+ but not *sly*− *S. suis* strains at a multiplicity of infection that was too low to cause suilysin-induced cell lysis. The authors suggested that suilysin mediates invasion of upper respiratory tract epithelial cells by virulent *S. suis* serotype 2 strains. Whether or not suilysin may be involved in invasion of (encapsulated and unencapsulated) *S. suis* in epithelial cells remains to be further elucidated.

Suilysin seems to contribute to the ability of virulent encapsulated serotype 2 strains to resist killing by porcine neutrophils in the presence of complete serum lacking specific IgG (Chabot-Roy *et al.*, 2006; Benga *et al.*, 2008). Addition of suilysin at sublytic concentration restored the phenotype of the isogenic *sly* mutant suggesting that some other mechanism in addition to cytotoxicity was involved (Chabot-Roy *et al.*, 2006). Our study indicated that expression of suilysin results in reduced internalization by porcine neutrophils but not reduced adherence to these cells (Benga *et al.*, 2008). No differences in the phenotype of the attenuated *sly* mutant were found between opsonizing and non-opsonizing conditions, in contrast to the work by Chabot-Roy *et al.* (2006). Only the latter is consistent with the speculation that suilysin leads to reduced complement deposition on the bacterial surface, as has been demonstrated for pneumolysin in the pathogenesis of pneumococci (Yuste *et al.*, 2005). Alternatively, suilysin might actively interfere with the ability of neutrophils to kill *S. suis*.

Dominguez-Punaro *et al.* (2007) investigated inflammatory reactions in the brain of CD1 mice after experimental infection with a suilysin producing serotype 2 strain. In agreement with a previous *in vitro* study (Graveline *et al.*, 2007), they detected upregulation of TLR 2, and to a lesser extent also TLR 3 but not TLR 4 mRNA. As discussed by the authors, this finding suggests a different mechanism of pathogenesis for suilysin in comparison to pneumolysin. Activation of TLR 4 by pneumolysin has been demonstrated to be crucial for a protective innate immune response against pneumococci (Malley *et al.*, 2003).

Expression of cytokines (IL-1, IL-6 and TNF-α) and chemokines (IL-8 and MCP-1) in a whole-blood culture system did not show significant differences between the wild-type strain and a suilysin knock-out mutant (Segura *et al.*, 2006). In earlier studies, this group had found that purified suilysin did not induce production of TNF-α and IL-6 by murine macrophages. In contrast, Lun *et al.* (2003) reported that suilysin triggered production of TNF-α in human monocytes and IL-6 production by pig pulmonary alveolar macrophages and monocytes. Furthermore, human endothelial cells secrete increased amounts of IL-6 and IL-8, but not MCP-1 after stimulation with purified suilysin at sublytic concentrations (Vadeboncoeur *et al.*, 2003). Sublytic concentrations of suilysin induced also transcription of the chemokine genes encoding IL-8 and MCP-1 in HEp-2 cells (our unpublished observations).

As multiple functions have been postulated for suilysin in interaction with host cells, severe attenuation of suilysin knock out mutants might be expected. However, attenuation in virulence of a suilysin knock-out mutant has only been demonstrated in mice (Allen *et al.*, 2001). In contrast, in piglets different isogenic suilysin serotype 2 mutants were as virulent as the wild-type (Lun *et al.*, 2003), or were only slightly attenuated in virulence (Allen *et al.*, 2001). On the other hand, the *sly* gene is more frequent among isolates from pigs with meningitis, septicemia and arthritis than in those from pigs with pneumonia (Staats *et al.*, 1999; King *et al.*, 2001; Silva *et al.*, 2006). Furthermore, geographical differences in prevalence of *sly*+ strains have been described. The most striking difference is the low prevalence of suilysin positive strains in North America in comparison to Europe and Asia (Gottschalk *et al.*, 1998).

Immunization with purified suilysin completely protected BALB/c mice against challenge with a highly virulent serotype 2 strain (Jacobs *et al.*, 1994). It is less clear whether suilysin is also a protective antigen in piglets. Challenge of three piglets immunized with purified suilysin suggested partial protection (Jacobs *et al.*, 1996, see also Table 4). However, we observed no protection against challenge with virulent serotype 2 and 9 strains after intranasal immunization with a live vaccine, though seroconversion against suilysin and suilysin neutralizing antibodies were elicited (Kock *et al.*, 2009).

In summary, suilysin is the only known secreted hemolysin of the CDC family in *S. suis*. Though suilysin is not essential for virulence of *S. suis* serotype 2 strains in pigs, *in vitro* experiments suggest that it may well have important functions in interactions with host cells.

Other hemolysins

Suppression subtractive hybridization analysis of the Chinese STSS strain HA9801 (used as tester) and the avirulent serotype 2 European strain T15 (driver) revealed, among other fragments, an ORF encoding a putative hemolysin type III. This DNA sequence was found in many virulent serotype 2 and 9 strains (Jiang *et al.*, 2009). Further ORFs encoding putative hemolysins are annotated in the genomes of the sequenced *S. suis* serotype 2 strains (Chen *et al.*, 2007, see also Table 3). However, at present there is no experimental evidence for expression of any other active hemolysin except suilysin.

Phospholipase C

Culture supernatants of *S. suis* serotype 2 strains induce degradation of phosphatidylcholine to diacylglycerol and choline phosphate (but not to phosphatidic acid),

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indicating that the tested strains secrete a phospholipase C (Jobin *et al.*, 2005a). Phospholipase C activity was identified in a search for potential factors in *S. suis* culture supernatants that are involved in the release of arachidonic acid from human BMECs observed in co-cultivation experiments with *S. suis* culture supernatants. Release of arachidonic acid was reduced in human BMECs treated with culture supernatants from a suilysin deficient mutant, but arachidonic acid was not released after treatment with suilysin. Based on these results the authors discussed a synergistic effect of suilysin and phospholipase C on the release of this important mediator by BMECs, which might play an important role in the pathogenesis of *S. suis*-associated meningitis.

EF

The 110-kDa EF encoded by the gene *epf* was identified as a protein associated with virulence in serotype 1 and 2 strains (Vecht *et al.*, 1991). The function of EF is still unknown. Isogenic *epf* mutants of serotypes 1 and 2 were as virulent as the wild-type in experimental infections indicating that EF is associated but not essential for virulence (Smith *et al.*, 1996). So far, EF producing strains have been identified only in serotypes 1, 2, 1/2, 14 and 15 (Wisselink *et al.*, 2000).

Large variants (>110 kDa) of EF (EF*) are expressed by some serotype 2 strains. They are characterized by long C-terminal tandem repeats (each 76 amino acids long). These repetitive sequences are missing in the 110 kDa EF. In contrast, the N-terminus, namely the first 811 amino acids, of EF and EF* are nearly identical. Based on the repeats five different classes of EF* proteins have been differentiated (Smith *et al.*, 1993). PCR assays are also available for differentiation of the different size variants of *epf* (Wisselink *et al.*, 2002; Silva *et al.*, 2006).

High mortality and morbidity was observed after experimental intranasal infection of newborn germfree piglets with MRP+ EF+ (referring to the 110 kDa protein) but not MRP+ EF* serotype 2 strains (Vecht *et al.*, 1992). This finding is contradictory to the high prevalence of MRP+ EF* serotype 2 (or the respective genotype) strains among invasive isolates in various epidemiological studies (Martinez *et al.*, 2003; Silva *et al.*, 2006). Furthermore, intravenous application of 10^8 CFU of an MRP+ EF* serotype 2 strain resulted in high mortality in piglets (our unpublished results).

Approximately 10% of the wild boars in Northwestern Germany carry *mrp*+ *epf** *cps*2 strains on their tonsils, whereas *mrp*+ *epf*+ (encoding the 110 kDa protein) *cps*2 strains were undetectable among these animals, in contrast to control domesticated piglets from the same region (Baums *et al.*, 2007). Therefore, we speculated that factors associated with modern swine production have led to the selection of the highly virulent MRP+ EF+ (110 kDa protein) serotype 2 strains. The high virulence of these strains might, however, be specific for domestic pigs as *S. suis* serotype 2 isolates from humans in Europe frequently express the large EF* variants, but not the 110 kDa EF protein (Smith *et al.*, 1993; Baums *et al.*, 2007).

Prevalences of various EF+ or EF* positive strains show substantial geographical variation. In contrast to Europe, MRP+ EF+ and MRP+ EF* serotype 2 strains have been detected only in single cases in North America (Gottschalk *et al.*, 1998).

The 110-kDa EF and the high molecular variants of EF are immunogenic proteins. Convalescent phase sera from animals infected either with MRP+ EF+ or MRP+ EF* serotype 2 strains generally contain high antibody titers against EF (our unpublished results). Immunization with EF alone did not elicit protection against MRP+ EF+ serotype 2 challenges, but a vaccine containing purified MRP and EF together with a water-in-oil adjuvant was as protective as a bacterin (Wisselink *et al.*, 2001). Nevertheless, a major problem of an EF-based vaccine is that many virulent *S. suis* strains, including all serotype 9 strains, do not express this factor.

Bacteriocin-like inhibitory substances (BLISs)

S. suis is not only an important invasive pathogen but also a successful colonizer of the upper respiratory, genital and alimentary tracts (Baele *et al.*, 2001; Su *et al.*, 2008). As part of such microbial ecosystems *S. suis* interacts with numerous other bacteria including other *S. suis* strains. Melancon and Grenier (2003) demonstrated production of BLISs in 4 of 36 tested *S. suis* strains, which were active against other *S. suis* strains and other bacterial species associated with pigs. These BLISs were sensitive to proteinase K, pronase and elastase indicating that they are proteinaceous. BLISs are thought to be of advantage for the producing strain to compete in a microbial ecosystem, but this has not been reported for *S. suis*.

Immunoglobulin G-binding protein

S. suis strains of various serotypes express at least one IgG-binding protein (Serhir *et al.*, 1993). Serotype 2 strains secrete a 52-kDa IgG-binding protein that reacts with a large variety of mammalian IgG and IgA molecules (Serhir *et al.*, 1995). The binding of IgG does not depend on the glycosylation state of the immunoglobulin molecule. The *S. suis* protein exhibits lower IgG-binding affinities than protein A and protein G, especially with regard to human IgG. In contrast to protein A and protein G, the *S. suis* IgG-binding protein also targets chicken IgG.

Concluding remarks

The primary host of *S. suis* is the pig, but it may also cause very severe diseases in humans. The high diversity of this

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pathogen is reflected by presence of 33 serotypes and numerous genotypes, and the emergence of different pathotypes in Europe, North America and Asia. As in many other bacteria, both surface-associated and secreted factors contribute to virulence and pathogenesis. Recent progress in 'omics' research has generated a large amount of data on putative virulence-associated factors, and recent outbreaks of human infections in Asia have enormously fostered such analyses. On the other hand, experimental evidence for their possible function and biological role in infection is available for only a few of these factors. Understanding epidemiology and pathogenesis as well as improving control strategies, in particular vaccination, requires knowledge on bacterial factors that is more than speculation based on sequence data, for example. Therefore, this review provided an overview on currently available information about *S. suis* factors, mostly proteins, with respect to their features and function as well as their possible significance for epidemiological typing, pathogenesis and vaccination strategies. The article demonstrated the substantial progress in the field, but also reminds us of the many unsolved questions and future challenges.

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