

The *Streptococcus suis* sortases SrtB and SrtF are essential for disease in pigs

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Abstract

The porcine pathogen *Streptococcus suis* colonizes the upper respiratory tracts of pigs, potentially causing septicaemia, meningitis and death, thus placing a severe burden on the agricultural industry worldwide. It is also a zoonotic pathogen that is known to cause systemic infections and meningitis in humans. Understanding how *S. suis* colonizes and interacts with its hosts is relevant for future strategies of drug and vaccine development. As with other Gram-positive bacteria, *S. suis* utilizes enzymes known as sortases to attach specific proteins bearing cell wall sorting signals to its surface, where they can play a role in host-pathogen interactions. The surface proteins of bacteria are often important in adhesion to and invasion of host cells. In this study, markerless in-frame deletion mutants of the housekeeping sortase *srtA* and the two pilus-associated sortases, *srtB* and *srtF*, were generated and their importance in *S. suis* infections was investigated. We found that all three of these sortases are essential to disease in pigs, concluding that their cognate-sorted proteins may also be useful in protecting pigs against infection.

INTRODUCTION

The porcine pathogen *Streptococcus suis* colonizes the upper respiratory tract of pigs, where it can reside as a commensal or invade and cause septicaemia, meningitis and arthritis. *S. suis*-associated disease places a substantial economic burden on the pig farming industry globally [1, 2]. It is also able to cross the species barrier from pigs to humans, with the zoonosis occurring through close contact with pigs and the consumption of undercooked pork or pork products [1, 3, 4]. The incidence of human disease, which was once sporadic, is now associated with larger outbreaks of disease, with the highest incidence of *S. suis* cases occurring in Asia [2].

S. suis produces a polysaccharide capsule, which covers the external surface of the bacterium. The capsule is an important virulence factor as it allows the organism to evade the host innate and adaptive immune systems [5–7]. Beneath the *S. suis* capsule is the Gram-positive cell wall composed of a thick peptidoglycan layer to which proteins are attached. Some *S. suis* surface proteins have also been shown to be important in pathogenesis, such as Fhb, which has

been shown to inhibit phagocytosis [8], and Ssu0587, which contributes to adhesion of the bacteria [9]. Many Gram-positive bacteria utilize enzymes known as sortases, which catalyze the covalent attachment of specific proteins to the cell wall peptidoglycan [10]. The proteins attached in this way are known as sorted proteins; they encode a C-terminal cell wall sorting signal (CWSS) consisting of an LPXTG-like motif, a hydrophobic domain and a positively charged tail. This signal is typically recognized by a type A housekeeping sortase, SrtA, which cleaves the protein between the T and the G amino acids of the LPXTG-like motif and transfers it to the cell surface by forming a covalent bond between the sorted protein and the peptidoglycan [11]. *S. suis* encodes a SrtA housekeeping sortase, the deletion of which significantly reduces the amount of protein on the cell surface of the bacteria [12] and attenuates its virulence in a piglet model of infection [13]. Interestingly, a *srtA* knockout mutant is as virulent as its parental strain in an intraperitoneal (IP) murine model of infection [14].

As well as SrtA, some Gram-positive bacteria encode secondary sortases, which have specialist functions. For example, type C sortases are responsible for the elongation of

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Two supplementary tables are available with the online version of the article.

polymeric pili structures on the cell surface [11]. Following nucleophilic attack on the T-G bond, SrtC enzymes catalyze the formation of isopeptide bonds between the LPXTG-like motif on one pilin protein and the surface lysine residue within the pilin specific YPKN motif on a second pilin protein, leading to elongation of the pilus structure [15]. Once the multimeric structure is assembled, SrtA performs the final transfer of the pilin subunits to the cell surface, terminating polymerization [11]. Depending on the strain, *S. suis* encodes up to six type C sortases [16–18] encoded in four distinct genetic loci. The *srtBCD* and *srtF* loci are associated with disease-causing isolates [18], the *srtG* cluster is absent from the serotype 2 model strain P1/7 and the *srtE* loci has been rendered non-functional by a number of point mutations within its coding sequence [18].

It is not unusual for Gram-positive bacteria to encode multiple class C sortases, each one assembling a cognate pilus [11]. However, where multiple class C sortases are encoded in the same locus, there is often redundancy in their function. The *srtBCD* locus encodes three type C sortases encoded in series, upstream of which there are four genes encoding hypothetical sorted proteins, *sbp1-4*. One of the putative sorted proteins, *sbp2*, is predicted to encode a major pilin and *sbp1*, an ancillary pilin [18], although a frame shift in *sbp2* renders it a truncated pseudogene (referred to herein as N-terminal *sbp2* or C-terminal *sbp2'*, as described previously) [18]. The truncated *sbp2'* encodes the Gram-positive secretion signal and YPKN motif, but is missing the C-terminal CWSS (found in *Spb2'*), therefore the truncated protein may be secreted but there is no mechanism by which it can be classically polymerized and covalently attached to the surface of the cell. Interestingly, the deletion of *sbp2'* attenuates both binding of the *S. suis* cells to the human laryngeal epithelial cell line Hep-2 and virulence in a zebrafish larvae model of disease [19], but with no CWSS present these phenotypes are likely to be independent of the actions of the sortases. Little is known about the *S. suis srtBCD* sortases and their putative substrates, and the role they play in *S. suis* pathogenesis is not yet understood.

The *S. suis srtF* locus has been more thoroughly characterized; it produces a pilus formed solely of the major pilin Sfp1, and a second ancillary pilin is encoded in the locus by *sfp2* but contains a frameshift rendering it a truncated pseudogene, the product of which does not feature in the pilus structure. SrtA is essential for transferring Sfp1 to the cell surface and SrtF is responsible for its polymerization into a pilus [20]. To date, no role for the *srtF* cluster in *S. suis* virulence has been identified; in the mouse sepsis model of disease mutants of *srtF*, *sfp1* and *sfp2* all behaved the same as the parental strain, and similarly adhesion and invasion of porcine brain microvascular epithelial cells (BMECs) was not impaired in these mutants [20]. These findings, however, do not rule out a role for SrtF in *S. suis* virulence in pigs.

In this study, we evaluate the role of the type C sortase genes *srtB* and *srtF* in the virulence of *S. suis* P1/7 strain by generating markerless in-frame deletion mutants of both genes and testing their pathogenesis first in the *Galleria mellonella* model of infection and then in pigs. We find that only SrtF is essential for disease in *G. mellonella*, but that both SrtB and SrtF are essential for *S. suis* to cause systemic disease in pigs, and while both mutants still colonized the tonsils of pigs their numbers were reduced compared to the wild-type parental strain P1/7.

METHODS

Bacterial strains and growth conditions

All strains and plasmids are presented in Table 1. The virulent serotype 2 strain P1/7 was used as the parental strain for mutagenesis and as the wild-type strain in subsequent experiments. *S. suis* was grown in brain heart infusion (BHI) broth (Oxoid) supplemented with 5 mg ml⁻¹ yeast extract and 0.1% L-cysteine (BHIS), with the addition of 1.5% agar (Bacto) for plates. Cultures were grown statically at 37°C in an atmosphere containing 5% CO₂. Where appropriate, media were supplemented with chloramphenicol (5 µg ml⁻¹). Plasmids were transferred into *S. suis* by electroporation as described previously [21]. For *G. mellonella* infections, exponential-phase cultures (OD 0.4–0.6)

Table 1. Strains and plasmids

Name	Detail	Source
<i>S. suis</i>		
P1/7	Wild-type; serotype 2	Pig, UK [33]
$\Delta cps2E$	In-frame deletion mutant of <i>cps2E</i> in strain P1/7	[22]
$\Delta srtA$	In-frame deletion mutant of <i>srtA</i> in strain P1/7	This study
$\Delta srtB$	In-frame deletion mutant of <i>srtB</i> in strain P1/7	This study
$\Delta srtF$	In-frame deletion mutant of <i>srtF</i> in strain P1/7	This study
<i>E. coli</i> top 10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 nupG recA1 araD139 Δ (<i>ara-leu</i>) 7697 <i>galE15 galK16 rpsL</i> (Str⁺) <i>endA1</i> λ⁻</i>	Invitrogen
pMTL82151	<i>E. coli</i> - <i>C. difficile</i> shuttle plasmid (pBP1; <i>catP</i> ; ColE1+ <i>tra</i>)	[34]
pAFP369	Construct for in-frame deletion of <i>srtA</i> in strain P1/7	This study
pAFP327	Construct for in-frame deletion of <i>srtB</i> in strain P1/7	This study
pAFP371	Construct for in-frame deletion of <i>srtF</i> in strain P1/7	This study

were harvested and stored in 20 % glycerol at -20°C . Bacterial suspension were thawed and washed with phosphate-buffered saline (PBS), and bacterial colony-forming units (c.f.u.) were quantified by plating on BHIS agar.

General molecular biology techniques

Plasmids were extracted using a plasmid miniprep kit (Qiagen) and genomic DNA was obtained by chelex extraction [cell pellets were vortexed in 5 % chelex (Sigma), boiled for 10 min and pelleted, and the supernatants were removed and used]. DNA was amplified for cloning using Phusion high-fidelity polymerase (NEB), and for screening using GoTaq polymerase (Promega), both in accordance with the manufacturers' instructions. DNA was extracted from PCR reactions and agarose gels using the QIAquick PCR and gel extraction kits (Qiagen), respectively. Plasmids were constructed by restriction/ligation cloning using restriction endonucleases, Antarctic phosphatase and T4 ligase (NEB) as per the manufacturer's instructions. Plasmids were confirmed by restriction analysis and Sanger sequencing (SourceBioscience).

Markerless in-frame deletion mutagenesis by allele exchange

Mutagenesis was performed in *S. suis* strain P1/7 and the markerless $\Delta srtA$, $\Delta srtB$ and $\Delta srtF$ mutants were constructed by the method previously described [22]. Briefly, the plasmid pMTL82151 was used as a *S. suis* suicide plasmid for allele exchange [22]. Primers were designed to amplify the DNA upstream and downstream of the *srtA*, *srtB* and *srtF* gene sequence, ensuring that the genes encoded upstream and downstream of *srtA* (*gyrA* and *radC*) and upstream of *srtF* (*ssu0249* and *murD*) were not amplified in their entirety in case their expression was lethal to the *Escherichia coli* cloning strain. Allele exchange cassettes were assembled by splicing by overlap extension PCR (SOE-PCR), digested with restriction endonucleases and ligated into pMTL82151 linearized using the same restriction endonucleases. A list of all primers and their corresponding restriction endonucleases can be found in Table S1 (available in the online version of this article).

Allele exchange plasmids were transferred to *S. suis* by electroporation as described previously [18] and transformants were grown on BHIS agar supplemented with chloramphenicol (Cm) to select for the plasmid borne *catP* gene integrated onto the *S. suis* chromosome. Single-crossover clones were sub-cultured daily on non-selective medium to allow a second recombination event to occur in which the plasmid borne *catP* marker could be lost. Double-crossover events were detected by replica-plating onto non-selective and Cm plates, and mutants were verified by PCR (Table S1) while the PCR products were checked by Sanger sequencing (Source Bioscience).

RNA isolation and RT-PCR

Overnight cultures of *S. suis* were sub-cultured at a 1 in 100 dilution and grown to either the mid-exponential or the early stationary phase. RNAprotect (Qiagen) was added

to the cultures at twice the culture volume once they reached the desired OD, and they were incubated at room temperature for 5 mins before the cells were pelleted and stored at -80°C until required. RNA was extracted by resuspending the pellets in FastRNA pro-blue solution (MPBio) and the cells were homogenized over a lysing matrix (B) in a Fastprep homogenizer (MPBio). Cell debris and the lysing matrix were removed by centrifugation and the supernatants were mixed with chloroform (Sigma); the phases were then separated by centrifugation and the upper phase was removed and mixed with 100 % ice cold ethanol and incubated for 3 h at -80°C to precipitate the nucleic acids, which were then pelleted by centrifugation, washed in 70 % ethanol and dried. Nucleic acid was then resuspended in nuclease-free H_2O and the nucleic acid concentration of the samples was measured.

To DNase-treat the nucleic acid, reactions were set up in a final volume of 150 μl which contained 30 μg nucleic acid (200 μgml^{-1}), 6 U DNase I (Ambion) and 3 μl RNasin (Promega) in DNase I buffer and were incubated at 37°C for 2 h. Subsequently, a further 6 U DNase I, 2 μl RNasin, 1 μl 10 DNase I buffer and 4 μl nuclease-free H_2O , was added to each reaction and incubated for a further 2 h at 37°C . The samples were cleaned up with the RNeasy kit (Qiagen) following the manufacturer's instructions. Successful removal of DNA was confirmed by PCR.

RNA was converted to cDNA using SuperScript III (Life Technologies) following the manufacturer's first-strand cDNA synthesis protocol. RT-PCR was carried out with the cDNA as a template and gDNA, RNA and H_2O as control templates for each PCR reaction.

Preparation of cell wall proteins

Overnight cultures of *S. suis* were harvested and resuspended in S buffer [20 mM Tris/HCl (pH 6.8), 10 mM MgCl_2 , 20 % raffinose) with 5000 U mutanolysin and protease inhibitor (Roche) to an OD ml^{-1} of 20. Suspensions were incubated for 2 h at 37°C before being harvested at 6000 g for 20 min. Supernatants containing the cell wall proteins were analysed by SDS-PAGE.

Western blot

Samples of isolated cell wall proteins were normalized according to the OD₆₀₀ of the cultures and a total volume of 15 μl was run on a Nu-Page 12 % Bis-Tris SDS-PAGE gel in MOPS running buffer (both Life Technologies). Proteins were transferred to an H+ membrane in a semi-dry blotter in transfer buffer (0.24 % Tris, 1.14 % glycine and 20 % methanol). The membrane was blocked in PBS plus 2 % milk (PBS-M) and probed with an anti-SlpF antibody (a generous gift from the Gottschalk laboratory) at a 1 in 1500 dilution for 1 h at room temperature. Following standard membrane washing with PBS-T (PBS plus 0.1 % Tween 20), a goat anti-rabbit secondary antibody conjugated to an infra-red dye (LI-COR) was added at a 1 in 10 000 dilution in PBS-M plus 0.1 % Tween 20 and 0.01% SDS and incubated in the dark for 1 h. The membrane was washed as

before and visualized using an infra-red imager (LI-COR Bioscience)

Biofilm assays

Overnight cultures of *S. suis* were sub-cultured to an OD of 0.05 in 2 ml BHIS in 24-well tissue culture plates in triplicate. Cultures were grown for 24, 48 and 72 h statically at 37 °C 5 % CO₂. Two millilitres of supernatant was aspirated and the wells were washed once with 1 ml PBS and dried for 10 min at 37 °C. Seven hundred microlitres of crystal violet (Sigma) was added to the wells and incubated statically at 37 °C for 30 min. Residual crystal violet was washed from the wells three times with PBS before biofilm-bound dye was solubilized in 700 µl 100 % EtOH for 30 min with shaking. Absorbance was read at 595 nm. Assays were repeated at least three times.

G. mellonella infections

Research-grade *G. mellonella* that were of uniform age and weight and free from antibiotic treatment were purchased from Biosystems Technology. *G. mellonella* were infected with a series of *S. suis* suspensions in PBS containing 10⁵, 10⁶ and 10⁷ c.f.u. of wild-type P1/7 and $\Delta cps2E$. Ten microlitre doses were infected subcutaneously into the hindmost proleg of 10 active *G. mellonella* larvae using a Hamilton syringe. Following injection, each group was placed in a separate 90 mm sterile Petri dish containing a 90 mm-diameter Whatman filter paper. *G. mellonella* larvae were then incubated at 37 °C and monitored for 24 h. Syringes were sterilized between injections of different strains with 70 % EtOH. Ten larvae were injected with sterile PBS and 10 larvae were left untreated. Death of larvae was distinguished by melanization of the larvae and cessation of movement. All experiments were repeated in triplicate independently. Once the optimal dose for comparison between the parental strain and the mutants was established as 10⁶ c.f.u., the larvae were infected with the parental strain P1/7 and the mutants $\Delta srtA$, $\Delta srtB$ and $\Delta srtF$ following the same procedures.

Pig infections

A total of 23 CDCD pigs (Struve laboratories) arrived in the laboratory at 35 days old; the study began when they were 71 days old. At day 0 nasal swabs were taken and 100 µl of undiluted sample was immediately plated on blood agar for *S. suis* culture, while the rest of the sample was frozen; blood samples were also taken and serum was removed and frozen. The pigs were then challenged via the intranasal (IN) route with 2 ml (1 ml per nostril) of approximately 10⁹ c.f.u. ml⁻¹ *S. suis* in PBS (Table 1). On days 2, 5, 7 and 9 and at necropsy nasal swabs were taken and 100 µl was cultured on blood agar and the rest of the sample was frozen. On day 5 and at necropsy tonsil swabs were also taken, of which 100 µl of undiluted sample was immediately plated on blood agar and the rest of the sample was frozen.

Throughout the study, pigs were observed for signs of severe disease, including lameness, lethargy and neurological symptoms. If presentation was severe enough (dyspneic, paddling and/or did not rise upon human entry into the

pen) the pigs were euthanized. If no clinical signs of disease were observed pigs were euthanized at day 15.

At necropsy gross lesions were recorded and samples were taken: blood from which serum was collected, tonsil swabs, swab of serosa (pericardium, thoracic cavity, abdominal cavity), joint tap or swab, cerebrospinal fluid (CSF) tap and lung lavage. Samples were collected in 2 ml PBS, except lung lavage, where 50 ml of PBS was instilled into the lung just above the tracheal bifurcation and aspirated with a pipette. A hundred microlitres of all of the samples was plated on blood agar plates and the rest was frozen.

Quantification of IgG by enzyme-linked immunosorbent assay (ELISA)

Serum IgG titres to *S. suis* were determined using an indirect ELISA. A sonicate of P1/7 grown overnight on 5 % sheep blood TSA plates was prepared and diluted in coating buffer (100 mM carbonate/bicarbonate buffer pH 9.6) to a final concentration of 1 µg ml⁻¹ of protein. Each well of Immulon-2 plates was coated with 0.1 ml and incubated overnight at 4 °C. The next day, plates were washed three times with PBS/Tween (0.05 %) and blocked with 0.2 ml of blocking buffer [2 % BSA in PBS/Tween (0.05 % Tween-20; PBS-T)] for 1 h at room temperature and then washed three times again. Serum samples taken on day 0 and day 15 from the pigs given the mutant strains were twofold serially diluted and incubated in duplicate at room temperature for 1 h. Plates were washed and *S. suis*-specific IgG was detected by adding 0.1 ml of anti-porcine IgG conjugated to horseradish peroxidase (KPL; catalogue 14-14-06; dilution 1:10 000) and incubating at room temperature for 1 h. Plates were washed and TMB substrate was added according to the manufacturer's recommendations (Life Technologies). After 15 min with substrate, 0.05 ml of stop solution (2N H₂SO₄) was added and the optical density was read at 450 nm with correction at 655 nm. The resulting absorbances were modelled using a non-linear function of the log₁₀ dilution using GraphPad Prism log (agonist) vs the response-variable slope four-parameter logistic model. Endpoints were interpolated by using twice the average absorbance value of gnotobiotic swine sera as the cut-off.

RESULTS

The *srtBCD* genes form a transcriptional operon with the putative sorted proteins

It is not known which proteins, if any, are sorted by the SrtB, SrtC and SrtD sortases of *S. suis* P1/7, but the primary candidates are the six open reading frames encoded upstream of the *srtBCD* genes. These putatively encode an ancillary pilin *sbp1*; a major pilin containing a frame-shift resulting in *sbp2'* and *sbp2''*; *sbp3*; *sbp4* and *ssu1890* (Fig. 1a). To determine whether these genes are transcribed, RT-PCR was carried out by amplifying short, internal regions within *sbp1*, *sbp2'*, *sbp2''*, *sbp3*, *sbp4* and *ssu1890*. The absence of PCR amplification products with RNA template and the presence of amplification with the cDNA for

all open reading frames confirmed transcription of all genes (Fig. 1b). To determine whether these genes are expressed as a transcriptional operon, RT-PCRs amplifying the regions between genes spanning the 3' region of one gene to the 5' region of the adjacent gene were carried out. As before, the presence of amplicons with the cDNA template and the absence of any product with RNA template confirmed that this genetic locus forms an operon, spanning *ssu1890* through to *srtD* (Fig. 1b)

Generation of sortase mutants by in-frame deletion

In order to characterize the *S. suis* sortases, markerless in-frame deletion mutants of the *srtA*, *srtB* and *srtF* genes were generated by allele exchange and confirmed by PCR and sequencing of the PCR products (Fig. 2a, Table 1) [22]. As *srtB* is encoded within an operon (Fig. 1b), it was particularly important that this mutation was markerless to reduce the likelihood of polar effects on the downstream genes *srtC* and *srtD*. We were able to confirm that transcription of *srtC* and *srtD* was intact in the $\Delta srtB$ mutant by RT-PCR of the *srtC-srtD* intergenic region (Fig. 2b). Growth curves were undertaken for all strains and no difference was seen in the growth kinetics between the wild-type and any of the mutants (data not shown).

Surface extractions were made of all three mutants and probed by Western blot with an anti-Sfp1 antibody. This antibody reacts to the major pilin protein of the SrtF cluster, Sfp1, and has previously been used to show that SrtA is essential for the addition of Sfp1 to the cell surface and that SrtF is essential for its polymerization into a filament. The $\Delta srtA$ and $\Delta srtF$ mutants generated in this study behaved as described previously; unsurprisingly, the $\Delta srtB$ mutant does not affect Sfp1 protein attachment and polymerization and appeared as the parental strain in the Western blot (Fig. 2c).

Biofilm formation increases in the absence of *srtF* and capsular polysaccharide

In *Streptococcus pneumoniae* biofilm formation has been found to reduce virulence in mice, although it does not reduce nasopharyngeal colonization [23]. This is due to a decrease in capsular polysaccharide and pneumolysin production, which confers quiescence during colonization [24]. Similarly, biofilm formation in *S. suis* causes a reduction in virulence due to the downregulation of virulence factors such as capsular polysaccharide and *mrp* genes [25]. As pili can be key in the ability of bacteria to form biofilms [26], we sought to determine whether mutation of the pilus-

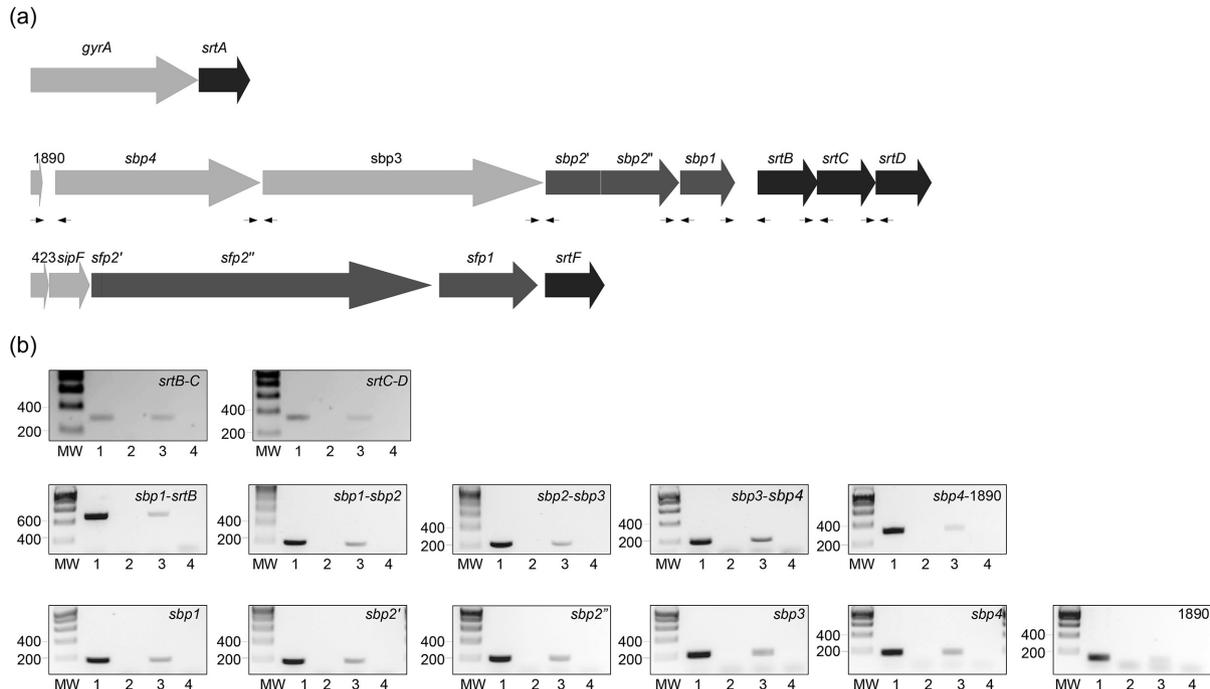


Fig. 1. Genetic organization of the type A and type C sortases and their associated genes in *S. suis*. (a) Schematic diagram illustrates the *srtA*, *srtBCD* and *srtF* genetic loci. The large arrows represent open reading frames; the dark grey ones are confirmed/putative sortases; the medium grey ones encode pili proteins; the light grey ones are genes encoding proteins with other functions. The small arrows represent primer pairs of the cross-junction RT-PCRs used to determine whether these genes formed the same transcriptional unit. (b) RT-PCR analysis revealed that all of the genes of the *srtBCD* locus are expressed (bottom row) and cross-junction PCRs indicated that they form a transcriptional operon (top and middle rows). MW, molecular weight marker; 1, gDNA; 2, RNA; 3, cDNA; 4, water control.

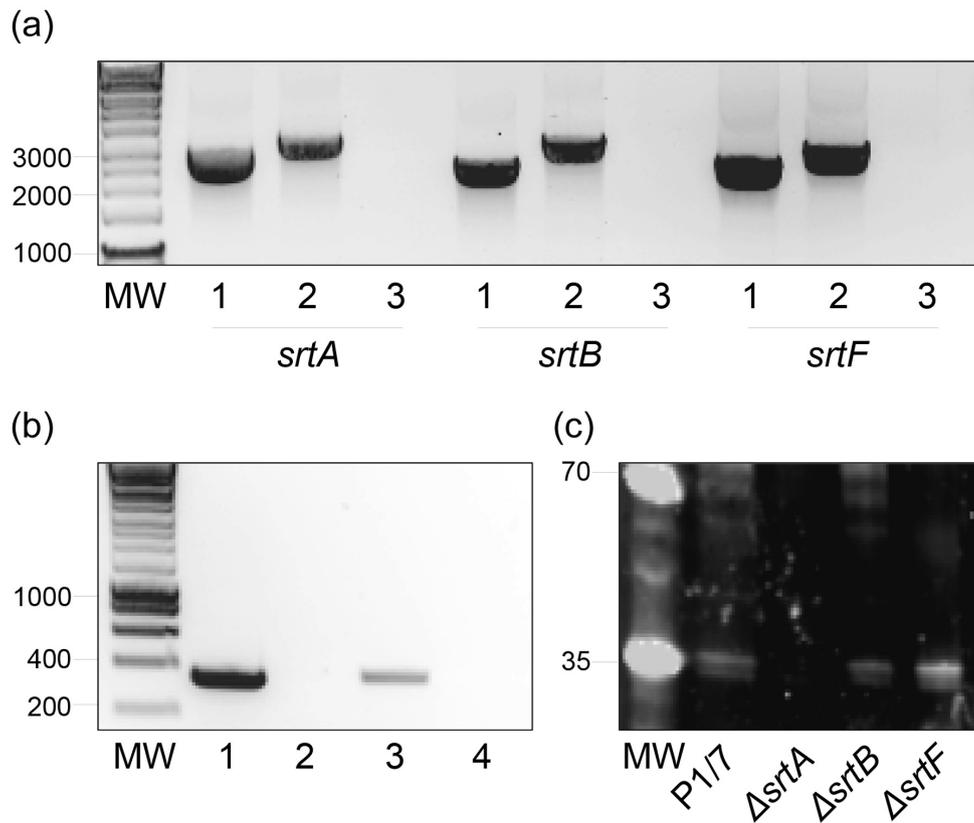


Fig. 2. Deletion of sortase genes. (a) PCR screening to confirm the $\Delta srtA$, $\Delta srtB$ and $\Delta srtF$ mutations initially identified by replica plating on chloramphenicol (Cm) and non-selective media; PCR was performed using primers to amplify across the deletion region. MW, hyperladder I molecular weight marker; 1, mutant DNA; 2, P1/7 *S. suis* genomic DNA; 3, water-only control. The gene name indicates the mutant analysed. (b) RT-PCR screening to confirm that the deletion of *srtB* did not affect transcription of the *srtC* and *srtD* genes downstream. MW, molecular WT marker; 1, gDNA; 2, RNA; 3, cDNA; 4, water control. (c) Cell surface extractions from P1/7 and the sortase mutants were probed with anti-Sfp1 antibody by Western blot (MW, molecular WT marker; gene names below indicate mutation). Sfp1 was identified in cell surface extracts of the wild-type and the $\Delta srtB$ mutants both as monomers and in a polymerized form. In the $\Delta srtF$ mutant monomeric Sfp1 was in the cell surface extracts but not in the polymerized form and the $\Delta srtA$ mutant.

associated sortases in *S. suis* had any effect on biofilm formation. Biofilm assays were carried out in microwell plates utilizing the wild-type P1/7 and the non-capsulated $\Delta cps2E$ mutant [22] as controls. We found that by 72 h, wild-type P1/7 had produced very low levels of biofilm, and we observed no differences in the $\Delta srtA$ and $\Delta srtB$ mutants (Fig. 3). Interestingly, the $\Delta srtF$ mutant produced approximately five and a half times more biofilm ($P=0.005$) compared with wild-type P1/7, though not as much as the $\Delta cps2E$ mutant, which produced approximately 27 times more than the wild-type ($P=0.0048$) (Fig. 3).

SrtF is essential for *S. suis* pathogenesis in the *G. mellonella* model of infection but SrtA and SrtB are not

It has previously been reported that *G. mellonella* larvae, which are generally found to be a useful model of the innate immune system, are suitable for studying *S. suis* pathogenesis [27]. As there is often variation in this

model, we first established it in our laboratory by testing for appropriate doses for infection of the wild-type P1/7 strain. Pathology occurs in a dose-dependent manner and the LD_{50} was determined to be 6.55×10^5 c.f.u. at 20 h post-infection (Fig. 4a). As an acapsular mutant has been shown previously to be attenuated in *G. mellonella* [27], we used an acapsular mutant $\Delta cps2E$ [22] as a second control for the model. We confirmed that the $\Delta cps2E$ mutant showed marked attenuation in the *G. mellonella* model, even at the highest infection dose tested when compared to the parental strain (Fig. 4b). When infected with the 10^6 c.f.u. of the parental strain, P1/7, survival was approximately 70% by 15 h, decreasing to around 30% by 20 h. With the $\Delta cps2E$ mutant however, survival was 100% at 20 h, significantly greater survival than the parental strain [Fig. 4B; $P=0.002$ by one-way analysis of variance (ANOVA)]. The LD_{50} for the $\Delta cps2E$ mutant was determined to be 2.66×10^8 ,

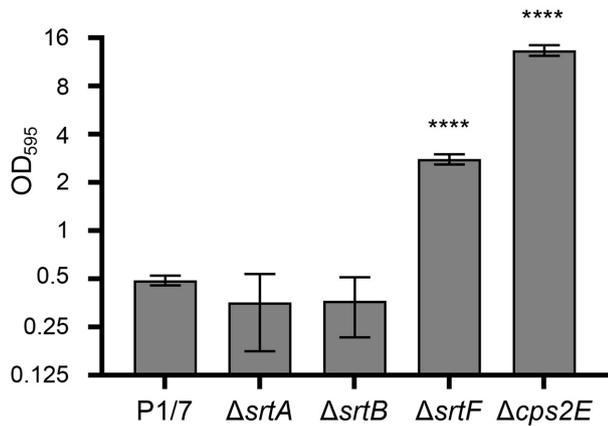


Fig. 3. Biofilm formation on an abiotic surface. The biofilm phenotypes of the mutants were investigated in their interaction with abiotic surfaces. Strains were grown in 24-well plates for 72 h and the relative amount of cells bound was measured with crystal violet stain. The amount of biofilm produced by the $\Delta srtA$ and $\Delta srtB$ mutants did not differ significantly from that produced by the wild-type, although the $\Delta srtF$ and $\Delta cps2E$ mutants produced significantly more biofilm in comparison with the wild-type ($P=0.005$ and $P=0.0048$, respectively, following one way ANOVA and a Dunnet multiple comparison test; **** indicates significance to three decimal places).

significantly higher than the parent P1/7 at 6.55×10^5 ($P=0.003$ by one-way ANOVA).

To determine whether any of the sortase mutants affected *S. suis* pathogenesis in *G. mellonella*, the larvae were infected with 10^6 c.f.u. of P1/7, $\Delta srtA$, $\Delta srtB$ and $\Delta srtF$ (a dose established as effective for comparison over 24 h). No significant difference was observed in the ability of the $\Delta srtA$ or $\Delta srtB$

mutants to cause disease compared to the parental strain over 24 h of infection (Fig. 4c). The $\Delta srtF$ mutant, however, showed significant attenuation in its ability to cause disease ($P=0.0039$), with 100 % survival in larvae infected with this mutant being observed (Fig. 4c).

Type C sortases are essential for efficient colonization and disease in the CDCD pig model of infection

The importance of the sortases in *S. suis* pathogenesis is inconsistent between different disease models. SrtA is essential for virulence in pigs but not in mice or *G. mellonella* [13, 14]; we found that SrtF is essential in *G. mellonella* disease but has previously been shown to be dispensable in mice [20], and here we found that SrtB is not essential in *G. mellonella* disease. As these mutations affect the cell surface architecture of the bacteria, we hypothesized that they are likely to be involved in colonization, adhesion and invasion of the host epithelia. Therefore IN pig infections should be the most appropriate model to assess the role of the sortases in the establishment and progression of *S. suis* infection in the nasopharynx of pigs.

Caesarean-derived colostrum-deprived (CDCD) pigs were used in this study. First, it was established that these animals were clear of *Streptococcus* contamination by culturing nasal swabs taken from the pigs. Animals were then challenged with approximately 10^9 c.f.u. of *S. suis* via the IN route (Table S2). The animals were observed at regular intervals for clinical signs of disease and euthanized as soon as symptoms met the criteria for severe.

Eight pigs were challenged with the wild-type parental strain P1/7 as a positive control and as a negative control five pigs were challenged with the non-capsulated $\Delta cps2E$ mutant [22], which has previously been shown to be

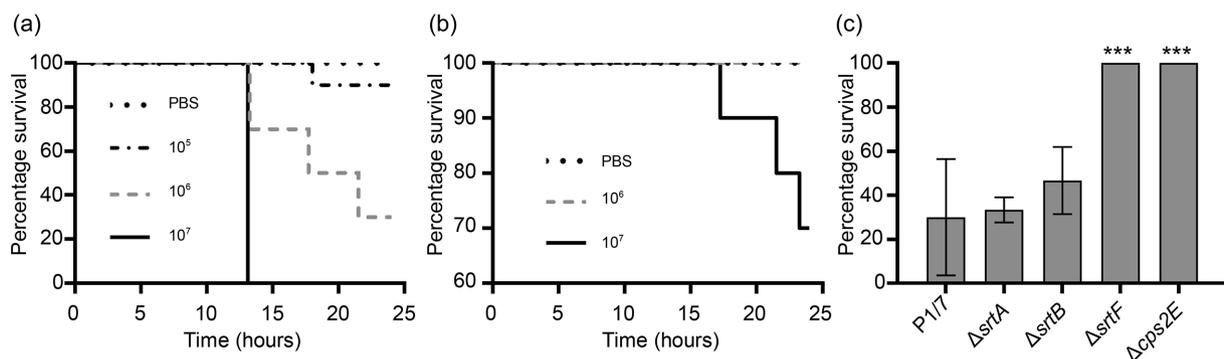


Fig. 4. The *S. suis* $\Delta srtF$ mutant is completely attenuated in virulence to *G. mellonella* larvae. (a) and (b) *G. mellonella* larvae were dosed with (a) 10^5 , 10^6 and 10^7 c.f.u. of P1/7 and (b) 10^5 and 10^6 c.f.u. of $\Delta cps2E$ and PBS control, and a suitable infectious dose was determined to be 10^6 for the comparison of the mutants with the wild-type. (c) *G. mellonella* larvae were infected with 10^6 c.f.u. of P1/7, $\Delta srtA$, $\Delta srtB$, $\Delta srtF$ and $\Delta cps2E$ and their percentage survival recorded at 24 h. The survival of *G. mellonella* infected with $\Delta srtF$ and $\Delta cps2E$ was 100 %, significantly higher than for those infected with the wild-type ($P=0.0039$ and $P=0.002$ respectively, following one-way ANOVA and a Dunnet multiple comparison test; *** indicates significance to three decimal places). The survival of *G. mellonella* infected with either $\Delta srtA$ or $\Delta srtB$ mutants was not significantly different from that for the wild-type.

avirulent in pigs [7]. To determine whether the type C sortases are important in disease pathogenesis in this model, five pigs were challenged with the $\Delta srtB$ mutant and five with the $\Delta srtF$ mutant (Table S2).

Seven of the eight pigs challenged with P1/7 showed clinical signs of disease and were euthanized by day eight of the study at the latest (Fig. 5 and Table 3). Symptoms included lameness, lethargy, tremors and a reluctance to rise (Table 3); some also displayed pathological symptoms of disease, such as swollen hocks and joints, while others had no remarkable gross or macroscopic pathology (Table 2). The eighth pig (994) displayed no clinical signs of disease for the duration of the study. Bronchoalveolar lavage fluid (BALF), serosal swabs, joint fluid, CSF and serum were plated at necropsy to determine whether the *S. suis* infection had become systemic. *Streptococcus* was cultured from at least two of the systemic sites of all seven pigs that succumbed to disease following infection with the P1/7 wild-type (Table 3). There was only one case in which *Streptococcus* was cultured from the BALF.

No systemic infection of *S. suis* was observed in animal number 994, the surviving animal. As expected from previous studies [5, 6], the $\Delta cps2E$ capsule mutant did not cause disease, no pigs challenged with this strain showed any signs of morbidity throughout the period of the study, none of the animals had to be euthanized and no *Streptococci* were cultured from the systemic sites at necropsy. However, between 50–100 *Streptococcal* colonies were grown from tonsil swabs from all five

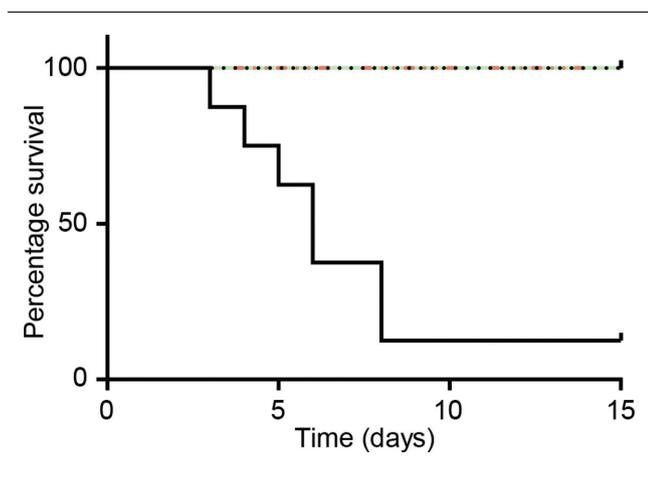


Fig. 5. The $\Delta srtB$ and $\Delta srtF$ mutants are attenuated in virulence in the pig model of infection. Pigs were infected with approximately 10^9 c.f.u. of *S. suis* at day 0. By day 8, seven of the eight pigs infected with P1/7 (—) had been euthanized due to the severity of the disease symptoms, while the remaining pig showed no signs of disease by the end of the study. All of the pigs infected with the $\Delta srtB$ (---), $\Delta srtF$ (· · ·) and $\Delta cps2E$ (●●●) mutants showed no signs of clinical symptoms for the duration of the study.

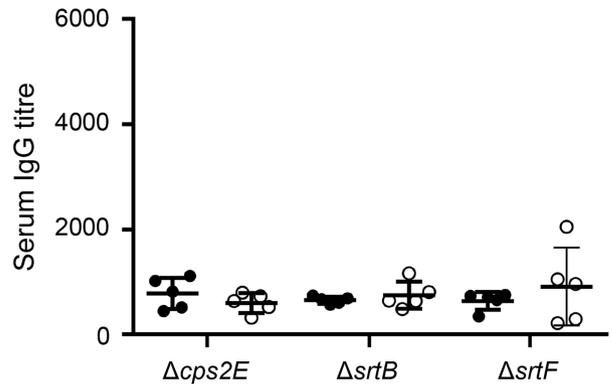


Fig. 6. IgG responses to the $\Delta srtB$, $\Delta srtF$ and $\Delta cps2E$ mutants do not explain their attenuation in pigs. (a) To determine whether *S. suis*-specific serum IgG was generated over the course of the pig infection study, an ELISA was carried out against P1/7 sonicate, probed with pig serum taken at day 0 (closed circles ●) and at day 15 (open circles ○) of the study. No change in *S. suis*-specific IgG antibody was seen in any of the pigs infected with the mutants over this time period.

pigs at day 5 post-infection, which appeared to be largely cleared by necropsy at day 15.

No symptoms of disease were observed in any of the pigs infected with either the $\Delta srtB$ or the $\Delta srtF$ mutant and all survived to the end of the study period (Tables 2 and 3). Similar to the $\Delta cps2E$ mutant, no *Streptococci* were cultured from the systemic sites at necropsy, but some bacteria were observed in swabs from the tonsils, between 22 and 72 c.f.u. for the $\Delta srtB$ mutant and between 6 and 26 c.f.u. (one animal with none) for the $\Delta srtF$ mutant on day five (data not shown). By day 15 no *Streptococcus* c.f.u. were isolated from tonsil swabs in three of the five pigs infected with $\Delta srtB$ and three of the five pigs infected with $\Delta srtF$, and in the four animals where they did grow the numbers were low (5–16 c.f.u.). Very few bacteria were grown from nasal swabs at this time and none were found in the blood. Therefore, the *srtB* and *srtF* mutants are able to colonize the tonsils, although to lesser extent than the parental strain, but it appears that they are not able to invade the host and become systemic.

No IgG antibody response in the pigs infected with the sortase mutants

We reasoned that the *srtB* and *srtF* mutants might be able to invade the host, but pathology or systemic infections could not be observed because the bacteria were being cleared more rapidly than the parental strain by either an adaptive or innate immune responses in the pigs. To determine whether an IgG antibody response had been generated in pigs infected with $\Delta cps2E$, $\Delta srtB$ and $\Delta srtF$, serum IgG titres from days 0 and 15 of infection were measured by ELISA against lysate of *S. suis* P1/7. We found that no or very little IgG antibody response to *S. suis* was observed in these animals, indicating that an IgG adaptive immune response to

systemic infection had not been generated (Fig. 6a). As this assay is specific for IgG, it does not exclude the possibility of other antibody isoforms having proliferated in response to *S. suis* during the course of the experiment, such as the mucosal antibody secretory IgA (sIgA).

DISCUSSION

Understanding the mechanisms of *S. suis* colonization in pigs and how this leads to systemic infection is important for tackling the disease in pigs and consequently in humans too by reducing or eliminating the reservoir of disease.

As cell surface proteins are often important colonization factors, here we investigated the involvement of two known sortases, the type A sortase SrtA and the type C sortase SrtF, and one putative type C sortase SrtB, in a number of *S. suis* phenotypes. We found that a mutation of *srtF* significantly increased biofilm formation compared to the wild-type and led to 100 % survival in the *G. mellonella* model of infection, phenotypes comparable with the capsular polysaccharide mutant *cps2E*. Neither the *srtA* nor *srtB* mutation had any effect on biofilm formation or survival in *G. mellonella*. Interestingly, both *srtB* and *srtF* mutants caused attenuation of virulence in the pig IN model of disease, a phenotype observed previously in a *srtA* mutant.

Unlike the *srtA* and *srtF* genes the role of the putative type C sortase genes *srtBCD* have not been studied in much detail. This locus contains the three putative sortases encoded alongside four putative sorted proteins, Sbp1–4, and we have been able to show that these genes form a single transcriptional unit or operon with the upstream gene Ssu1890 and that our in-frame deletion of *srtB* did not knock out the expression of *srtC* and *srtD* (Fig. 1). Another example of multiple sortases encoded in the same locus is the PI-1 pilus locus of *S. pneumoniae* (sometimes called the *rlrA* cluster), which encodes three sortase genes *srtC1*, *srtC2* and *srtC3*, a major pilin *rrgB*, two ancillary pilins *rrgA* and *rrgC*, plus a regulator *rlrA* [28]. There is redundancy in the action of these sortases, but SrtC1 is both necessary and sufficient for wild-type pilus formation and localization [28, 29].

Table 2. Clinical/pathological findings

Animal	Clinical findings	Pathological findings
1–15	NCS	No significant lesions at necropsy
990	D5 lame on rear legs and reluctant to rise	Swollen hocks, cloudy joint fluid
991	D4 lame on R rear, lethargic, tremors	Swollen R hock, cloudy joint fluid
992	D8 depressed, reluctant to rise, cyanotic ears	Swollen hocks, cloudy joint fluid
993	D3 uncoordinated, reluctant to rise	No significant lesions at necropsy
994	NCS	No significant lesions at necropsy
995	D8 lame, tremors, reluctant to rise	No significant lesions at necropsy
996	D5 lame on front and rear legs	Swollen joints, cloudy joint fluid
997	D6 side paddling	No significant lesions at necropsy

NCS, no clinical signs; D, day; R, right.

For the majority of sorted proteins encoded in the *S. suis* genome the canonical LPXTG sortase motif is utilized, including the proteins Sao and MRP and the SrtF-associated pilin Sfp1, which have all been shown to be absent from the cell surface in a *srtA* mutant [13]. The putative sorted proteins of the *srtBCD* operon, however, encode a non-standard LPXTG motif (Table 4). The first amino acid of the motif in Sbp1 and Sbp2'' and Sbp3 is not the typical leucine, but is instead either an isoleucine (Sbp1 and Sbp2'') or a tyrosine (Sbp3). The motif of the fourth putative sorted protein in the cluster, Sbp4, has an LYKTG sequence encoding a standard leucine at the first position but a tyrosine instead of a proline at the second position. Interestingly, this is similar to the sorted proteins of the PI-1 pilus locus of *S. pneumoniae*, which also contain non-standard LPXTG motifs in which the first amino acid is not the canonical leucine but is instead either a tyrosine (RrgA), an isoleucine (RrgB) or a valine (RrgC). These sorted proteins transfer to the cell surface by the action of their cognate type C sortases rather

Table 3. Culture of *Streptococcus* from systemic sites at necropsy

Animal	Onset of clinical signs/necropsy (days)	BALF	Serosal swab	Joint fluid	CSF	Serum
990	5/6	1	tntc	tntc	tntc	148
991	4/4	–	–	tntc	–	tntc
992	8/8	–	2	tntc	tntc	17
993	3/3	–	–	10	tntc	95
994	NCS/15	–	–	–	–	–
995	8/8	–	10	tntc	tntc	tntc
996	5/5	–	–	tntc	–	20
997	6/6	–	–	–	tntc	72

NCS, no clinical signs; tntc, too numerous to count.

Table 4. Pentapeptide cell wall sorting signals of different proteins

Protein					
Canonical	L	P	X	T	G
Sfp1	L	P	S	T	G
Sbp1	I	P	Y	T	G
Sbp2'	I	P	Q	T	G
Sbp3	Y	P	K	T	G
Sbp4	L	Y	K	T	G
<i>S. pneumoniae</i> PI-1 pilins	Y/I/V	P	X	T	G

than the housekeeping SrtA and it is thought that the variation in the CWSS impairs their recognition by SrtA. [29, 30]. It is possible that the SrtBCD sortases of *S. suis* also perform the covalent attachment to the peptidoglycan independently of SrtA.

We found that SrtB is essential to disease in CDCD pigs, even when both SrtC and SrtD are still present and (perhaps more interestingly) despite the fact that the putative major pilin of the loci contains a frameshift that prevents it from forming a functional sorted protein. The other putative sorted proteins encoded within the locus are therefore implicated in invasion of the epithelia by *S. suis* and consequently in disease, and so they are worthy of further study.

The SrtF-associated pilus is dependent on SrtA for its attachment to the cell surface and SrtF for the polymerization of the pilin subunits. It was notable that the *srtF* mutant caused a large increase in biofilm formation and completely attenuated virulence in the *G. mellonella* model of infection, but the *srtA* mutation did not. It is possible that the pleiotropic effects of the *srtA* mutant counteract the impact that the absence the SrtF pilus alone has. The results in *G. mellonella* suggest that the deletion of *srtF* renders the bacteria more susceptible to clearance by the innate immune system and suggests increased sensitivity to the antimicrobial peptides of the innate immune system. Both the attenuation in *G. mellonella* and the increase in biofilm formation may be related to altered charge on the surface of the bacterium [31].

As with the *srtB* mutant, we found that SrtF is required for virulence in the CDCD pig IN infections. This is interesting as it has previously been found that SrtA is likewise essential for wild-type virulence in IN pig infections but redundant in mouse IP infections. Our findings indicate that there is a specific role for SrtF pilus in pig IN infections, which is not required in mouse IP infections.

Bacterial surface proteins, including pili, are often important for specific host interactions, such as adhesion and invasion of host epithelial cells, playing a crucial role in initial colonization but not pathogenesis in systemic infections [10]. As the sortase mutants affect the cell surface protein composition, they may also affect specific host-pathogen

interactions. Here it seems that they are essential for the colonization of the host (as observed in IN pig infections) but not for systemic infection (as observed in IP mice infections).

Our findings suggest that contrary to previous conclusions the type C sortases SrtB and SrtF are necessary for wild-type levels of colonization and invasion of the upper respiratory tract of pigs. This both adds to our knowledge of *S. suis* pathogenesis and suggests that generating an immune response to Sfp1 and/or the putative sorted proteins of the *srtBCD* cluster may be useful in protecting against *S. suis* infection in swine. We have also further demonstrated that while alternative models of infection are useful they have limitations and show disparities from the natural host of *S. suis* and should be interpreted with caution. The advantages and disadvantages of all of the animal models currently used to study *S. suis* have been thoroughly discussed previously [32].

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Animal studies were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals and the Guide for the Care and Use of Agricultural Animals in Research and Teaching. The animal experiments were approved by the USDA National Animal Disease Center's Institutional Animal Care and Use Committee

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