Use of proteins identified through a functional genomic screen to develop a protein subunit vaccine that provides significant protection against virulent *Streptococcus suis* in pigs

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Running title: Development of a *Streptococcus suis* vaccine for pigs

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Abstract

Streptococcus suis is a bacterium commonly carried in the respiratory tract that is also one of the most important invasive pathogens of swine, commonly causing meningitis, arthritis, and septicemia. Due to the existence of many serotypes and a wide range of immune evasion capabilities efficacious vaccines are not readily available. The selection of *S. suis* protein candidates for inclusion in a vaccine was accomplished by identifying fitness genes through a functional genomics screen and selecting conserved predicted surface-associated proteins. Five candidate proteins were selected for evaluation in a vaccine trial and administered both intranasally and intramuscularly with one of two different adjuvant formulations. Clinical protection was evaluated by subsequent intranasal challenge with virulent *S. suis*. While subunit vaccination with the *S. suis* proteins induced IgG antibody titers to each individual protein, a cellular immune response to the pool of proteins, and provided substantial protection from challenge with virulent *S. suis*, the immune response elicited and degree of protection were dependent on the parenteral adjuvant given. Subunit vaccination induced IgG reactive against different *S. suis* serotypes indicating a potential for cross-protection.

Introduction

Streptococcus suis is a Gram-positive bacterium commonly carried in the tonsil and nasal cavity of swine that can cause systemic disease and secondary pneumonia, especially in young pigs. Streptococcal disease is widespread wherever pig production occurs and systemic invasion most commonly results in septicemia, meningitis, arthritis, and/or polyserositis causing significant economic losses to the industry. *S. suis* is also a zoonotic agent capable of causing meningitis in humans, and although historically sporadic in nature, there have been recent larger

outbreaks in China and Vietnam with high levels of mortality (1-3). There are at least 33 capsular serotypes (1-31, 33 and 1/2) of *S. suis*, with serotypes 32 and 34 reassigned (4), and ongoing controversy over the appropriate speciation of serotypes 20, 22, 26, and 33 (5). In most countries, capsular serotype 2 is the most virulent and the most frequently isolated from both diseased swine and humans (6). However, depending on geographic location other serotypes such as 1, 1/2, 3, 7, 8, 9, 14 are commonly isolated from diseased pigs (7-10).

The mechanisms that enable *S. suis* to invade systemically from the respiratory tract are not well understood, though numerous potential virulence factors or virulence-related factors have been identified (reviewed in Segura et al.) (11). However, none of these factors appear individually to correlate completely with the ability to cause disease and thus virulence is probably multifactorial, and, to date, no highly effective vaccines have been developed to protect against *S. suis* disease. Genomic analysis of large numbers of isolates with known commensal or disease-associated provenance revealed a complex population structure with high levels of recombination and marked genomic differences between the two groups (12). The presence of multiple serotypes and high genotypic variability may make it difficult to develop broadly protective vaccines.

A relatively new technique called TraDIS (Transposon Directed Insertion Sequencing) or TnSeq is a method used to simultaneously identify bacterial fitness genes by the generation of a random transposon library disrupting individual gene expression and assessment of the effects of the disruption on survivability under selection conditions. High throughput sequencing technology is used to generate sequence reads spanning the transposon/chromosome boundaries of each insertion, allowing for the *en masse* accurate mapping of transposon insertion sites (13-17). By identifying members of the library that are no longer present after the applied negative

selection, disrupted genes that are important for fitness under the applied conditions can be readily identified. Prior to this study we processed a strain P1/7 *S. suis* TraDIS library through an *in vitro* organ culture system (IVOC) using pig nasal epithelium to select genes encoding proteins that may be involved in colonization fitness. Using *in silico* bioinformatics approaches five *S. suis* proteins were further selected on the basis of likely cell surface location and conservation. The five proteins were cloned, expressed and purified in *Escherichia coli* and then tested as potential vaccine candidates in swine.

Results

Characteristics of the five candidate vaccine proteins. Five candidate vaccine proteins (SSU0185, SSU1215, SSU1355, SSU1773, SSU1915) were selected based on the results of the experimental functional genomics screening and *in silico* bioinformatics approaches described in the Materials and Methods section (Table 1). Candidates with a significant reduction in fitness of transposon mutants in IVOC with swine respiratory epithelium were narrowed down to genes encoding surface-associated proteins excluding those containing trans-membrane domains in the middle of protein coding sequence (Table 1). Homology searches were used to identify proteins highly conserved in 459 publically available *S. suis* genomes which cover all serotypes with the exception of 20, 22 and 33 and come from Argentina, Canada, China, Denmark, Germany, The Netherlands, United Kingdom and Vietnam (Table 2 and 3). Of the five proteins chosen, SSU0185 and SSU1355 were found in the genome of all 459 *S. suis* isolates, SSU1915 was found in >99% of the isolates, and SSU1215 and SSU1773 were found in >98%, of the isolates (Table 2). Protein identities of the five subunit vaccine candidates were compared to *S. suis* strains with complete genomes in GenBank (Table S1) and disease-associated *S. suis* serotype

representatives from the 459 *S. suis* genome collection (Table 3). These strains represent disease-associated *S. suis* serotypes isolated from diverse global geographic sources. Overall, the five candidate proteins had >91% protein identities in these strains compared to those in P1/7. The immunoreactivity of the recombinant proteins was tested with serum, collected from a convalescent pig infected with a serotype 2 *S. suis* strain under experimental conditions, in a Western blot (Figure 1). Reactivity to four of the proteins (SSU1215, SSU1355, SSU1773, and SSU1915) was observed. The potential to apply the five candidate proteins as a pool of subunit vaccines has not been previously published, patented or tested in pig protection studies.

Parenteral adjuvant formulation and boosting significantly impacts the serum IgG *S. suis* protein specific response. Two groups of pigs were vaccinated with the five proteins both intranasally with Polyethyleneimine as adjuvant and intramuscularly with one of two adjuvant combinations, AddaVax/Carbopol (group 1) or Emulsigen-D (group 2) as described in the Materials and Methods section (Table 4). Groups 3-5 were control groups given PBS mixed with the same adjuvants given to groups 1 and 2 or PBS only, respectively. Overall, serum IgG antibody reactive against all five proteins was detected in all vaccinated pigs, and there was an anamnestic response after administration of the boost vaccination (Figure 2). No *S. suis* protein-specific IgG was detected in the pigs given adjuvant alone or PBS (data not shown), nor was there a response detected in serum collected at day 0. Two weeks following priming (day 14), IgG titers specific to individual *S. suis* proteins were significantly higher in serum from pigs in group 2 (Emulsigen-D adjuvant) compared to group 1 (Carbopol/AddaVax adjuvant) and this trend continued after the response was boosted (day 21 and 28). In fact, IgG titers to the proteins in group 2 pigs after a single injection were approximately equal to the titers in group 1 pigs after 2 injections.

Peripheral *S. suis* protein-specific IFN-γ recall response declines following boost immunization. The number of PBMCs producing IFN-γ following re-exposure to the pool of *S. suis* proteins was used as a measure of vaccine-induced cell-mediated immunity. The number of IFN-γ secreting cells (SC) following re-stimulation with *S. suis* proteins was greatest on day 14 post-priming, and adjuvant formulation had a significant impact on responses with pigs in group 2 (Emulsigen-D adjuvant) having significantly higher numbers of IFN-γ SC compared to group 1 (Carbopol/AddaVax adjuvant) (Figure 3). The number of IFN-γ SC detected decreased over time; with an average of 263 and 32 IFN-γ SC for group 2 detected on days 14 and 28, respectively. PBMC collected from pigs in groups 3, 4 and 5 (no antigen groups) did not have more than 13 IFN-γ SC detected at any time point following stimulation *S. suis* proteins. In addition, the number of IFN-γ SC detected following stimulation with media alone remained below 10 at each time point evaluated. While there was, on average, an increase in the number of IFN-γ SC using PBMC from pigs in group 1 at day 14 post-priming, it was not significantly increased over control groups (groups 3-5).

Cytokines produced by PBMCs following restimulation with the protein pool were highest in pigs vaccinated with Emulsigen-D adjuvant. PBMCs collected on day 28, 2 weeks after boost vaccination, were stimulated with the pool of five *S. suis* proteins as another measure of vaccine-induced cell-mediated immunity. Overall, cytokines produced by PBMCs following restimulation with the protein pool were highest in pigs from group 2 (Emulsigen-D adjuvant) (Figure 4). These levels were statistically higher for group 2 compared to all other groups for IL-2 and TNF-α, whereas there was no statistical difference in the amount of these cytokines produced among groups 1 (Carbopol/AddaVax adjuvant) and 3-5 (control groups).

Subunit vaccination provides significant protection against lethal challenge with *S. suis* and is associated with the immune response and adjuvant given. Following virulent challenge, nine out of ten pigs in non-vaccinated control groups 3-5 developed severe signs of systemic *S. suis* infection (lameness with swollen joints, anorexia, depression, dyspnea, and neurologic signs) and had to be euthanized (Figure 5). *S. suis* was cultured from systemic sites of these 9 pigs including serosa (5/9), joint (9/9), CSF (9/9), and spleen (8/9), and macroscopic and microscopic lesions consistent with *S. suis* infection including meningitis, polyserositis and arthritis were present. *S. suis* was readily isolated from the nasal cavity and tonsil of these pigs as well, but only small numbers of *S. suis* were isolated from the lung lavage of 5 of them, and pneumonia was not a prominent lesion that was seen. There was one pig in group 5 that only developed intermittent mild lameness beginning 1 day after challenge that continued throughout the observation period but demonstrated no other clinical signs, and *S. suis* was only isolated from the nasal wash and tonsil of this pig at the termination of the experiment on day 15.

By comparison, the two vaccinated groups had 3/6 pigs in group 1 (Carbopol/AddaVax adjuvant) and only 1/6 pigs in group 2 (Emulsigen-D adjuvant) develop severe systemic disease requiring euthanasia (Figure 5). Survival was significantly greater for group 2 compared to the combined non-immunized control groups. Similar to the control groups *S. suis* was isolated from systemic sites (4/4 serosa, 4/4 joint, 3/4 CSF, and 3/4 spleen) of the four pigs in the vaccinated groups that had to be euthanized and macroscopic and microscopic lesions consistent with *S. suis* infection were present. The nasal cavity and tonsil were heavily colonized in all the vaccinated pigs, but virtually no *S. suis* was isolated from the lung lavage from any of these pigs. One pig from group 2 was lame for two days with no other clinical signs and recovered uneventfully, and *S. suis* was only isolated from the nasal wash and tonsil but no systemic site of this pig, and no

macroscopic or microscopic lesions consistent with *S. suis* infection were present at the end of the experiment when all the remaining pigs were euthanized. In addition, *S. suis* was isolated from the spleen from one pig in each of group 1 and 2 that appeared clinically healthy throughout the experiment. Neither of these pigs had any macroscopic or microscopic lesions consistent with *S. suis* infection.

Subunit vaccination induces IgG reactive against whole *S. suis* bacteria. An indirect ELISA was performed to determine if serum IgG from vaccinated pigs collected on day 28 post-vaccination reacted with whole P1/7 *S. suis* bacteria or other *S. suis* isolates representing serotypes commonly associated with disease (serotypes 1, 2, 1-2, 3 and 14). Although there were some differences in the degree of reactivity across the different isolates, there was an appreciable IgG response to all *S. suis* isolates tested, indicating a considerable amount of reactivity to different isolates of *S. suis*, which vary in respect to serotypes (Figure 6). As with the other measured immune parameters, the *S. suis*-specific IgG response induced in group 2 (Emulsigen-D adjuvant) pigs was higher than that in group 1 (Carbopol/AddaVax adjuvant).

Discussion

The five *S. suis* proteins in this study were chosen based on first determining genes/proteins that were predicted to play a role in fitness during colonization of the respiratory tract, the initial stage in establishing infection, using a respiratory epithelium IVOC system and transposon mutant library. The identified proteins are predicted to have functions in several physiological processes, in particular those associated with metabolism and nutrient acquisition, which might explain their role in survival on respiratory epithelium.

SSU0185 was identified as a putative tagatose-6-phosphate aldose/ketose isomerase. The ortholog of this protein, AgaS, is believed to be part of the pathway for utilization of the amino sugar, N-acetyl-D-galactosamine in *E. coli* (18). The abundance of free sugars is scarce in the respiratory tract and mucins, a major component of the mucus produced by respiratory surfaces, contain glycoproteins composed of sugars, amino sugars, and sulphated sugars commonly linked to a protein core via an N-acetylgalactosamine (19). Orthologs of *agaS* have been identified in other *Streptococcus* species, such as *Streptococcus pneumoniae*, where it was shown to be upregulated upon exposure to human macrophage-like cells and when grown in the presence of mucin, potentially explaining the importance of this protein for survival in the respiratory tract (20, 21).

SSU1915 was identified as a putative maltose/maltodextrin-binding protein whose ortholog is MalX, a lipid-anchored solute binding protein of an ATP binding cassette (ABC)-transporter. MalX has been reported as a streptococcal virulence factor involved in carbohydrate metabolism, specifically in polysaccharide degradation and synthesis (22). Members of the *mal* regulon of *Streptococcus pyogenes* have been shown to enhance colonization of the oropharynx through their niche-specific role in the utilization of dietary starch (23-25). Another study identified *malX* of *S. pneumoniae* as one of the niche-specific virulence genes upregulated in the lung and confirmed attenuation of virulence of a *malX* mutant during lung infection (26). In the same report, vaccination with MalX induced high antibody titers but not significant protection in an intraperitoneal challenge model (26). In contrast, Moffitt et al. demonstrated that intranasal vaccination with the *S. pneumoniae* protein SP2108, the MalX ortholog, was protective in a mouse model of pneumococcal nasopharyngeal colonization (27). Subsequently they established that the lipid modification of this protein is critical to its immunogenicity in a TLR2-dependent

manner, and there was an *in trans* effect of the lipoprotein that enhanced the immunogenicity of a co-administered nonlipidated antigen (28).

SSU1355 was identified as a putative surface-anchored 5'-nucleotidase, a hydrolytic enzyme that catalyzes the hydrolysis of a nucleotide into a nucleoside and a phosphate. These enzymes have been identified as virulence factors, purportedly by hydrolyzing extracellular nucleotides for purine salvage, degrading nucleotide diphosphate sugars that can then be used by the cell, and/or by generating extracellular adenosine in the host, which is a powerful immunosuppressant signaling molecule. *Staphylococcus aureus* produces extracellular adenosine to evade clearance by the host immune system, an activity attributed to the 5'-nucleotidase activity of adenosine synthase (AdsA) (29).

SSU1215 was identified as a putative surface-anchored dipeptidase. These enzymes play roles in several physiologic processes, such as catabolism of exogenously supplied peptides and the final steps of protein turnover.

SS1773 was identified as a putative surface-anchored serine protease. Prokaryotic serine proteases have roles in several physiological processes, such as those associated with metabolism, cell signaling, and defense response and development; however, functional associations for a large number of prokaryotic serine proteases are relatively unknown.

Since the methods used to identify these proteins indicated they were involved in respiratory colonization fitness, there was the possibility that locally induced mucosal or parenterally induced systemic immune responses, or both, would be important for protection. Since raising CDCD pigs is not a trivial matter and *S. suis* infection can have severe clinical consequences, it was decided to vaccinate with all five proteins by both routes to enhance the potential for success using the fewest number of pigs initially. Subsequently, further experiments

could be conducted to determine the role of each of the proteins and the role of the route of delivery in protection, and test protection against a heterologous challenge. Polyethyleneimine, an organic polycation, was chosen as the adjuvant for intransal vaccination because it has previously been shown to be a potent mucosal adjuvant for delivery antigens of mucosal pathogens (30, 31). We chose a combination of Addavax™, a squalene-based oil-in-water adjuvant similar to MF-59® used in human influenza vaccines in Europe, and Carbopol®-971, a polyanionic carbomer as one choice for parenteral adjuvant based on previous work demonstrating this type of combination yielded an additive or potentially synergistic adjuvant effect (32). In addition, we chose Emulsigen®-D, an oil-in-water emulsion with dimethyldioctadecylammonium bromide as the second parenteral adjuvant, which has also been shown to induce enhanced immune responses compared to some commonly used adjuvants (33). Both the magnitude of the systemic immune response and degree of protection was dependent on the parenteral adjuvant administered with the proteins. This would suggest that parenteral vaccination was the important delivery method for protection; however, a role for mucosal immunization in protection or priming of the immune response cannot be ruled out, and additional studies separating the routes of administration will be needed to determine these roles.

Even though the proteins were identified as potentially contributing to fitness for respiratory colonization, all surviving vaccinated animals showed tonsil and nasal colonization by the challenge organism. A quantitative comparison of colonizing bacterial load for immunized versus non-immunized animals was beyond the scope of this preliminary study, so there could have been a reduction of numbers of *S. suis* colonizing the respiratory sites that was not detected. In addition, since mucosal IgA was not measured it is difficult to state whether there was a failure of induction of mucosal antibodies to these proteins or a failure of antibodies

to prevent colonization. The impact of immunization on reduction of colonization load by pneumococcus in a mouse model was found to be dependent on individual host as well vaccine associated factors (34). There was a reduction of systemic disease in vaccinated animals, which could be due to reduced colonization and invasion or an increase in bactericidal/opsonic antibodies, or both. *Streptococcus suis* was also isolated from the spleen of two apparently healthy vaccinated pigs. These animals probably had an ongoing bacteremia that was being controlled and cleared by the immune response since, as indicated, the animals showed no antemortem, post mortem or histopathological signs of streptococcal disease. It is possible that this represented a very recent bacteremia; however, in our infection model with this strain of *S. suis*, we rarely have pigs develop or succumb to disease past day 10 of exposure.

Peripheral IFN-γ recall responses were evaluated at various time points after vaccination, and there was a reduction in the number of peripheral *S. suis*-specific IFN-γ SC after the boost (Figure 3). However, there was an increase in peripheral *S. suis*-specific IgG levels after the second dose of vaccine, indicating a boost in immune responses following the second dosing. While the reduction in IFN-γ SC was somewhat unexpected, it is important to note that IFN-γ SC serve as a single measure of immune cell activation, and cell-mediated immune responses after prime-boost were likely skewed towards T-helper responses not involving IFN-γ production. Given the increased levels of *S. suis*-specific IgG after the boost, T cell responses were likely directed towards B-cell affinity maturation and plasma cell generation, which would include production of IL-13 and IL-5, though levels of these cytokines were not measured in this study. Overall, subunit vaccination with the five *S. suis* proteins induced an immune response that provided substantial protection from lethal challenge with virulent *S. suis*, and specifics on the mechanism of protection warrant further investigation.

S. suis is a diverse species of multiple serotypes, each represented by immunologically different capsule types, and displaying a wide range of immune-evading features that, to date, has challenged the development of efficacious vaccines (35). In particular, although opsonizing antibody is believed to be key to *S. suis* killing in infected animals (36), the antibody response to *S. suis* capsule has been shown to be limited in infected animals (37). Although much effort has already focused on subunit candidates, especially surface associated targets (reviewed by Baums et al.) (38), recent reports emphasize the ongoing challenges of matching candidates with promising measures of protection in mouse models and *in vitro* assays with *in vivo* survival outcomes in live challenged pigs (39).

The five proteins identified are highly conserved and present in almost all strains of *S. suis* tested including probable non-virulent strains. Since these strains are normal colonizers of pigs, one might expect that antibodies against these proteins are already present in pigs on farm. There was reactivity to four of the proteins in serum collected from a convalescent pig infected with virulent *S. suis* (Figure 1); however, non-virulent strains are commensal microbes that could colonize without triggering a significant immune response. The diversity of antibody responses to these proteins in pigs naturally exposed to *S. suis*, with or without disease, might shed further light on their respective contribution to immune protection. Further studies will also be needed to evaluate the optimum approach to field application of these subunits as protective immunogens, including the potential for sow versus piglet immunization and the possibility of prior passive or active antibody interference. In addition, the reactivity of the sera from vaccinated pigs against several diverse *S. suis* strains commonly associated with disease in pigs may indicate a potential for cross-protection that will have to be confirmed through further challenge studies.

Materials and methods

Bacterial strains, vectors, media and antibiotics used in the study. Bacterial strains and vectors used in this study are listed in Table 5. *S. suis* strains were routinely grown at 37 ^{o}C in Todd-Hewitt broth (Oxoid) supplemented with 0.2% yeast (Sigma) (THY) or on Columbia agar (Oxoid) containing 5% (v/v) defibrinated horse blood (TCS Bioscience) (CBA). *E. coli* strains were routinely grown at 37 ^{o}C on Luria Bertani (LB) agar plates or cultured in LB broth (Oxoid). *E. coli* strains expressing recombinant proteins were grown at 37 ^{o}C in 2YT broth (Life Technologies). Kanamycin (Sigma) at the concentration of 100 µg/ml was used to select *E. coli* transformants. All the strains were stored at -80 ^{o}C in 20% glycerol.

S. suis (P1/7), a serotype 2 isolate from the blood of a pig with meningitis (40), was used for challenge and was grown on tryptic soy agar containing 5% sheep blood (Becton, Dickinson and Co.) at 37 ^{o}C overnight, scraped from the plates and resuspended in phosphate buffered saline (PBS) to an optical density of 0.42 at A_{600} to give an inoculum dose of 1 x 10^{9} cfu/ml. Each challenged pig received 1 ml per nostril (2 ml total).

General molecular biology techniques. The genomic DNA of *S. suis* strains was isolated using MasterPure™ Gram positive DNA purification kit (Epicentre Biotechnologies). Bacterial lysates of *S. suis* were prepared using Instagene™ Matrix, a Chelex-based resin (Bio-Rad Laboratories Ltd.) according to the manufacturer's instructions. The plasmid DNA samples were prepared using a QIAprep Spin Miniprep Kit (Qiagen) or a HiSpeed Plasmid Maxi Kit (Qiagen). Plasmids and genomic DNA were stored at -20 °C.

The polymerase chain reactions (PCRs) for screening bacterial colonies were set up with Go Taq Green Master Mix (Promega Ltd.) according to the manufacturer's instructions. The amplification conditions used were as follows: initial denaturation at 95 ^{o}C for 2 minutes

321 followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds
322 and extension at 72 °C for a period determined by the size of the PCR product (1 minute/kb),
323 with a final extension step at 72 °C for 7 minutes.

324 The PCR products used for cloning were amplified using Phusion® High Fidelity DNA
325 polymerase (Thermo-Fisher Scientific) according to the manufacturer's instructions. The
326 reactions contained 100 ng of template DNA or 1-5 µl bacterial lysate, 200 µM of each dNTP
327 (Bioline Ltd.), 0.5-1 µM of each primer (Sigma-Aldrich Ltd.), 1× PCR buffer, 1 unit of DNA
328 polymerase and DMSO at a final concentration of 3% when required. The initial denaturation
329 was done at 98 °C for 30 seconds followed by 30 cycles of denaturation at 98 °C for 10 seconds,
330 annealing at appropriate temperatures for 30 seconds and extension at 72 °C for a period
331 determined by the size of the PCR product (10-30s/kb). The final extension was done at 72 °C
332 for 7 minutes.

333 The primers used in this study are listed in Table 6. The primers were designed using
334 Primer3web version 4.0.0 (http://primer3.ut.ee) and synthesized by Sigma-Aldrich Ltd. The
335 primers were rehydrated with deionized water to a concentration of 100 µM on arrival and
336 working stocks of 10 µM concentration were prepared. All primers were stored at -20 °C.

337 The PCR products and DNA samples were analyzed by agarose gel electrophoresis. The
338 agarose gels were visualized and photographed using the Gel Doc™ XR+ imaging system with
339 Image Lab™ image acquisition and analysis software (Bio-Rad Laboratories Ltd.).

340 SDS-PAGE analyses were performed with whole cell lysates or purified proteins. Samples
341 were diluted in equal volumes of 2X SDS sample buffer, heated at 70 °C for 10 minutes and run
342 on 4-12% (v/v) Bis-Tris gels (Life Technologies) to confirm protein expression.

Selection of candidate vaccine proteins. A strategy of combining experimental functional genomics screening (IVOC with TraDIS) with *in silico* bioinformatics approaches was applied for selection of candidate vaccine proteins using a library generated in *S. suis* strain P1/7 (13-17, 41). The selection consists of the following steps: (1) candidate fitness genes (defined as a gene that harbored at least one transposon insertion mutant with significant reduction in fitness in a swine respiratory epithelium IVOC system) were determined through previous functional genomics screening, (2) protein subcellular localization was predicted *in silico* with bioinformatics approaches using PSORTb (http://db.psort.org/) and LocateP (http://www.cmbi.ru.nl/locatep-db/cgi-bin/locatepdb.py) databases or based on literature mining to shortlist fitness genes encoding surface-associated proteins [(cell wall anchored or extracellular (lipid-anchored or secretory)], (3) proteins containing transmembrane domains in the middle of protein coding sequence were excluded, (4) *in silico* protein homology based searches to identify proteins with cross-protection potential: i.e. the presence of the protein from the *S. suis* P1/7 genome was used as a query in a BlastX search and we identified proteins present (80% identity over 80% of the length) in 459 publically available strains or in the majority of disease-associated strains (12), (5) a final pool with five potential candidate vaccine proteins were chosen whose potential to be applied as a cassette of subunit vaccine has not been previously published, patented or tested in pig protection studies.

Cloning and expression of candidate vaccine proteins. Genes of interest were cloned from the genome of *S. suis* strain P1/7 excluding the signal sequences when present. Signal peptide cleavage sites of open reading frames (ORFs) were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP). The PCR products of candidate genes were cloned in to the pET-30 Ek/LIC vector (Merck Millipore) and fusion plasmids were transformed into *E.*

coli NovaBlue (Merck Millipore) according to the manufacturer's instructions. The positive recombinants were confirmed by PCR and DNA sequencing and then transformed into *E. coli* BL21 (DE3) (Merck Millipore) for expression. Overnight culture of *E. coli* BL-21 (DE3) strains carrying the recombinant plasmids were used to inoculate fresh 1-6 L 2YT broth and grown to OD_{595nm} 0.6 at 37 °C in broth supplemented with 100 µg/ml kanamycin, then induced with 1mM IPTG (isopropyl β-D-1-thiogalactopyranoside, Sigma) at 37 ^{o}C for 2, 4 and 24 hours. Protein expression was checked by SDS-PAGE using whole cell lysates.

Purification of recombinant vaccine proteins. Recombinant proteins were purified from 1-6 L cultures grown in 2YT broth and induced with 1 mM IPTG for 2 to 4 hours. Cell pellets were washed once in PBS and centrifuged at 3,000 × *g* for 15 minutes. The cell pellets were resuspended in binding buffer (10 mM imidazole, 300 mM NaCl, 50 mM phosphate, pH:8.0) and sonicated on ice for 6 minutes. Appropriate amounts of Benzonase and rLysozyme (Novagen, Merck Millipore) were added to reduce the viscosity of the lysate and improve protein extraction efficiency. The lysates were first centrifuged at 3,000 × *g* for 10 minutes at 4 °C to pellet debris and the supernatants were subjected to further centrifugation at 75,000 × *g* for 1.5 hours at 4 °C. Recombinant proteins were subjected to purifications by nickel His-Tag affinity chromatography, anion exchange chromatography, CHAP chromatography and gel filtration when appropriate. Target proteins were confirmed by peptide mass fingerprinting. Protein concentration was determined using spectrophotometry and purified proteins were stored at -80 °C.

Immunoreactivity of the recombinant proteins with convalescent pig sera. Immunoreactivity against the purified recombinant proteins was tested using serum from a conventionally-reared pig experimentally infected with *S. suis* serotype 2. Naïve sera for a

control was a pool collected from Gottingen mini-pigs (Serolabs Ltd.), which were reared in a pathogen-free environment and not expected to have any antibodies against *S. suis*. The purified recombinant proteins were separated on 4-12% (v/v) Bis-Tris gels under denaturing conditions and transferred to PVDF membranes. The membranes were rinsed in Tris buffered saline (30 mM tris base, 138 mM NaCl, 2.7 mM KCl, pH 8.0) with 0.05% Tween 20 (TBST) and then blocked with 2% casein in TBST, overnight at 4 ^{o}C. The pig sera (1:2000) were used as primary antibody and Horseradish-peroxidase (HRP) conjugated goat anti-pig (1:10000) (Sigma) was used as secondary antibody. The primary and secondary antibodies were diluted in 1% casein in TBST and membranes were probed at room temperature (RT) for 1-1.5 hours. The blots were then washed three times in TBST for 10 minutes at room temperature. The membranes were developed with Chemiluminescent substrate (Novex® ECL substrate reagent kit, Life Technologies) according to the manufacturer's instructions. The ECL substrate treated membranes were exposed to X-ray film (Amersham Hyperfilm ECL, GE Healthcare Lifesciences) for a suitable duration and developed in an X-ray film developer.

Vaccine protection study. The USDA-ARS-National Animal Disease Institutional Animal Care and Use Committee approved all animal work. Twenty-two, 5-week-old, Caesarean-derived, colostrum-deprived (CDCD) pigs were distributed into groups as follows (Table 3): group 1 pigs (6 pigs) were given a 2 ml dose of vaccine containing 250 µg protein (50 µg per subunit) with 1ml of Addavax^{TM} emulsion (Squalene-based oil-in-water adjuvant-Invivogen), and 5 mg of Carbopol$^{®}$-971 (Lubrizol Corporation) intramuscularly (IM) in the neck and a 2 ml dose of vaccine containing 500 µg protein (100 µg per subunit) and 500 µg of Polyethyleneimine (Sigma) intranasally (IN- 1 ml per nostril); group 2 pigs (6 pigs) were vaccinated similarly IN but in the 2 ml IM dose the proteins were mixed with Emulsigen$^{®}$-D (oil-

412 in-water emulsion with dimethyldioctadecylammonium bromide – MVP technologies) at a 1:5
413 (v/v) mix; groups 3 and 4 were control groups given PBS mixed with the same adjuvants given
414 to groups 1 and 2 respectively (3 pigs each); and group 5 was given PBS only (4 pigs). Pigs
415 received a booster dose of the same respective formulation 2 weeks after priming, and 2 weeks
416 after the boost pigs were challenged with 2 ml of 10^9 CFU/ml *S. suis* P1/7 IN (1 ml per nare).
417 Blood was collected on day 0 (prime) for serum, and days 14 (boost), 21 (one week post-boost)
418 and 28 (challenge) for serum and peripheral blood mononuclear cells (PBMC) to evaluate
419 vaccine immunogenicity. After challenge pigs were observed for clinical signs of disease
420 (approximately every 4-5 hours except for an 8 hour overnight period), including lameness,
421 lethargy, and neurological symptoms. If presentation was severe (such as neurologic
422 involvement, severe lameness, or depression that resulted in recumbency with reluctance to
423 stand) the pig was euthanized. Pigs not showing signs of disease or only transitory or mild signs
424 of disease were euthanized 15 days post challenge. At necropsy nasal wash, swabs of serosa and
425 hock joint (or other affected joint), cerebrospinal fluid (CSF), lung lavage, and a section of tonsil
426 and spleen were collected for culture. Nasal turbinate, tonsil, lung, heart, kidney, liver, spleen,
427 retropharyngeal lymph node, brain and synovium were collected for microscopic pathological
428 examination.

429 **Evaluation of the humoral immune response to vaccination.** Serum IgG titers to
430 individual *S. suis* proteins and reactivity to inactivated P1/7 were determined using an indirect
431 ELISA. Blood was collected into a BD Vacutainer Serum Separator Tube (SST) and serum
432 isolated according to manufacturer's recommendation (BD Pharmingen) with storage at -80 °C
433 until used in assays. For evaluation of antibody titers to individual *S. suis* proteins Immulon-2
434 plates were coated with 0.1 ml of each individual protein in 100 mM carbonate-bicarbonate

buffer (pH 9.6) overnight at 4 °C at the following concentrations: SSU1773 (1 µg/ml), SSU1355 (2 µg/ml), SSU1915 (1 µg/ml), SSU0185 (1 µg/ml), SSU1215 (0.5 µg/ml). The next day, plates were blocked with 0.2 ml of blocking buffer [2% BSA in PBS tween (0.05% Tween-20; PBS-T)] for 2 hours at RT and then washed three times with PBS-T. Eleven, two-fold serial dilutions of serum (starting at 1:2000) collected from each pig were made in 1% BSA/PBS-T, transferred to the ELISA plate in duplicate and incubated at RT for 2 hours. Plates were washed and *S. suis* specific IgG detected by adding 0.1 ml of anti-porcine IgG conjugated to horseradish peroxidase (KPL, catalog 14-14-06, dilution 1:10,000) and incubating at RT for 1 hour. Plates were washed and TMB substrate added according to manufacturer's recommendations (Life Technologies). After 15 minutes with substrate, 0.05 ml of stop solution (2N H_2SO_4) was added and optical density read at 450 nm with correction at 655 nm. The resulting OD data were modeled as a nonlinear function of the Log_{10} dilution using Graph Pad Prism (La Jolla, CA) log (agonist) vs. response-variable slope four-parameter logistic model. Endpoints were interpolated by using 4X the average OD of the day 0 sample of each respective pig serum as the cutoff.

To determine whether serum IgG reacted with whole P1/7 *S. suis* bacteria, heat-inactivated (HI) P1/7 was used as antigen in an indirect ELISA. To make antigen, a single P1/7 colony was inoculated into 5 ml THB and incubated at 37 °C in 5% CO_2 at 200 rpm for approximately 6 hours, at which time it had reached an OD=0.6 at Abs600. The bacteria were centrifuged at 4000 x g to pellet, media decanted and bacteria resuspended in 5 ml PBS. Bacteria were heat-inactivated (HI) by incubating the suspension in a water bath at 85 °C for 20 minutes. Inactivation was confirmed by plating 0.1 ml of the heat-inactivated preparation on blood agar plates and incubating the plates at 37 °C in 5% CO_2. No growth was observed on the plate after 2 days. Aliquots were stored frozen at -80 °C. Protein concentration of the HI P1/7 was determined

using BCA protein microtiter assay according to manufacturer's recommendations (Pierce). Immulon-2 plates were coated with 0.1 ml of 7.5 µg/ml of HI P1/7 diluted in 100 mM carbonate-bicarbonate buffer (pH 9.6). Serum samples collected on day 0 and day 28 from each pig were diluted 1:500 and used in the assay. P1/7-specific IgG was detected and the ELISA completed as described above for individual proteins. Data is reported as the OD at 450 nm with correction at 655 nm. A checkerboard of HI P1/7 concentrations and a pool of sera from day 0 and day 28 was used to determine optimal ELISA conditions (data not shown). Similar techniques were used to evaluate IgG reactivity with a collection of other HI *S. suis* strains comprised of two randomly selected representatives of those serotypes most commonly associated with disease (1, 2, 1/2, 3 and 14) (see Table 1), with bacteria reaching OD's of 0.6 - 1.1 at 600 nm in the 6-8 hour culture period prior to HI (data not shown) and all HI *S. suis* coated at 7.5 µg/ml for the ELISA.

Evaluation of the cell-mediated immune response to vaccination. To evaluate induction of cell-mediated immunity following vaccination, ELISpot assays were performed to enumerate IFN-γ-secreting cells following *in vitro* stimulation with a pool of the vaccine proteins. Blood was collected by venipuncture into a BD Vacutainer Cell Preparation Tubes (CPT) with sodium citrate for isolation of PBMC using culture media as previously described (42). PBMC were enumerated and seeded at $2.5x10^5$ cells per well in the IFN-γ ELISpot plates in duplicate for each treatment. PBMC were stimulated with a protein pool in final volume of 0.25 ml (1 µg/ml of each individual protein per well). Control wells received media alone or pokeweed mitogen (0.5 µg/ml). Approximately 18 hours after stimulation the ELISpot assay was completed according to manufacturer's recommendations (R&D Systems, Minneapolis, MN). Spots were enumerated using a S5UV ImmunoSpot instrumentation and software (Cellular Technology Ltd., Shaker Heights, OH) and data analyzed using GraphPad Prism software (La

Jolla, CA). The count for duplicate wells for each treatment for each pig was determined and used to calculate the mean for each group.

Cytokines produced by PBMCs collected on day 28 following restimulation with the protein pool were also measured. PBMC culture supernatants were collected 72 hours after restimulation with the protein pool or media-only and used to evaluate cytokine levels secreted by the cells. The amount of IFN-γ, TNF-α, IL-2, and IL-10 in the media was determined by multiplex cytokine ELISA according to manufacturer's recommendations using provided recombinant proteins as standards to determine concentrations in the supernatants (Aushon Biosystems)

Statistical Analysis. Survival analysis was performed using the product limit method of Kaplan and Meier, and comparing survival curves using the logrank test (GraphPad Prism, La Jolla, CA). Antibody titers were Log10 converted and a two-tailed student's t-test was used to evaluate statistical differences between groups 1 and 2 for indicated comparisons, with a p-value <0.05 considered significant. One-way analysis of variance (ANOVA) with a Tukey's multiple comparison post-test was performed to evaluate statistical differences between groups ($p<0.05$) for the number of IFN-γ secreting cells and cytokine production. Graph Prism software (version 6.0) was used for statistical analysis.

Acknowledgements

The authors would like to thank Steven Kellner, Sarah Shore, Zahra Olson, and Lilia Walther for their excellent technical assistance; Dhaarini Raghunathan and Shaowen Li for their contribution to protein purification; and Nate Horman, Jason Huegel and Tyler Standley for their excellent animal care. This work was supported by a Longer and Larger (LoLa) grant

from the Biotechnology and Biological Sciences Research Council (BBSRC grant numbers BB/G020744/1, BB/G019177/1, BB/G019274/1 and BB/G018553/1), the UK Department for Environment, Food and Rural Affairs, and Zoetis (formerly Pfizer Animal Health) awarded to the Bacterial Respiratory Diseases of Pigs-1 Technology (BRaDP1T) consortium. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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660 Vaccine Immunol 19:1722-1729.

661 Table 1. Characteristics of the five candidate vaccine proteins

Antigen encoding genes	Function/ortholog	Range of TraDIS fitness scores[a]	Full length protein residues (AA)	N-terminal signal peptide[b] (AA)	Protein subcellular localization prediction[c]	Conserved Domain	Fusion protein[d] AA / KDa
SSU0185	Putative tagatose-6-phosphate aldose/ketose isomerase (AgaS)	-4.66 to -8.58 (3/3)	389	/	Extracellular (literature mining)	/	432 /47.4
SSU1215	Putative surface-anchored dipeptidase	-0.90 to -10.22 (3/4)	607	1-27	Cell-wall anchored (*in silico*)	LPSTG	623 / 67.4
SSU1355	Putative surface-anchored 5'-nucleotidase	-0.81 to -8.23 (3/4)	674	1-30	Cell-wall anchored (*in silico*)	LPNTG	687 / 74.1
SSU1773	Putative surface-anchored serine protease	1.00 to -8.7 4 (6/8)	1692	1-40	Cell-wall anchored (*in silico*)	LPQTG	1695 / 187.4
SSU1915	Putative maltose/maltodextrin-binding protein precursor (MalX)	-5.03 to -5.05 (2/2)	419	/	Lipid-anchored (*in silico*)	/	462 / 49.0

662 [a] TraDIS fitness scores were presented as $\log_2$ fold change of Output:Input determined by
663 DESeq2 after normalisation. The fraction of significantly attenuated mutants in each gene is
664 shown in parentheses, using the parameters: input read ≥ 500, P- value ≤ 0.05.
665 [b] Genes encoding the surface proteins were cloned without the N-terminal signal peptides.
666 [c] *in silico* protein subcellular localization predictions by PSORTb and LocateP
667 [d] The amino acid residues and molecular weights of pET30 Ek/LIC fusion proteins were
668 calculated including the protein tag generated from the vector (43 AA, 4.8KDa) and excluding
669 the signal peptides if present.
670
671 Table 2. Presence of the five immunogenic antigens in 459 isolates of *S. suis*

Protein	Presence in *S. suis* isolate collection[a]			
	No. of isolates in which protein is present	Clinical[b] (292 isolates)	Non-clinical[c] (134 isolates)	Not Known[d] (33 isolates)
SSU0185	459	100%	100%	100%
SSU1215	452	99%	97%	94%
SSU1355	459	100%	100%	100%
SSU1773	450	98%	97%	100%
SSU1915	458	100%	99%	100%

672 [a] The presence of the protein was investigated by taking the sequence of the protein from P1/7
673 and using BlastX against the 459 genomes. If the protein had an 80% identity over 80% of the
674 length, it was classified as present.
675 [b]Isolates recovered from either systemic sites in pigs with clinical signs and/or gross pathology
676 consistent with *S. suis* infection (including meningitis, septicaemia and arthritis) or respiratory
677 sites in the presence of gross lesions of pneumonia from the lung were classified as clinical.
678 [c] Isolates from the tonsils or tracheo-bronchus of healthy pigs or pigs without any typical signs of
679 *S. suis* infection but diagnosed with disease unrelated to *S. suis* (such as enteric disease or
680 trauma) were classified as non-clinical.
681 [d] Isolates for which there was insufficient information about the pigs sampled were classified as
682 not known.

683 Table 3. Protein identities of the five subunit vaccine candidates in disease-associated *S. suis*

684 serotype representatives[a]

Strain ID	Serotype	SSU0185	SSU1215	SSU1355	SSU1773	SSU1915
SS021[b]	1	100%	99%	100%	100%	100%
SS045	1	100%	100%	100%	100%	100%
SS100	1/2	100%	100%	100%	100%	100%
SS043	1/2	98%	99%	99%	98%	100%
SS002	2	100%	100%	100%	100%	100%
SS008	2	98%	99%	99%	98%	100%
SS053	3	98%	99%	99%	97%	100%
SS084	3	98%	99%	99%	97%	100%
SS062	4	97%	90%	99%	96%	97%
SS079	4	88%	43%	75%	NP[c]	85%
SS018	7	98%	99%	99%	98%	99%
SS024	7	98%	99%	99%	98%	99%
SS068	8	98%	99%	99%	98%	100%
SS091	8	98%	99%	99%	97%	100%
SS015	9	97%	91%	96%	96%	97%
SS088	9	97%	90%	96%	96%	97%
SS078	10	96%	100%	95%	96%	97%
SS063	14	100%	100%	100%	100%	100%
SS077	14	100%	100%	100%	100%	100%
SS097	16	98%	98%	97%	97%	98%
SS037	22	88%	43%	73%	NP	85%
SS009	23	97%	91%	96%	96%	97%
SS082	31	98%	92%	96%	97%	97%

685 [a]The panel contains 2 representatives (where possible) of disease associated serotypes.

686 Respiratory isolates are selected where no other systemic isolate was available.

687 [b]Strains in bold also used in cross reactive ELISAs shown in Figure 6.

688 [c]NP = not present, if the protein had less than an 80% identity over 80% of the length, it was

689 classified as not present.

690 Table 4. Experimental groups.

Group	Vaccine/Adjuvant/Route	Challenge	Number of Pigs
Group 1	*S. suis* proteins/Polyethyleneimine/IN	*S. suis* P1/7	6
	S. suis proteins/Carbopol® & AddaVax™/IM		
Group 2	*S. suis* proteins/ Polyethyleneimine/IN	*S. suis* P1/7	6
	S. suis proteins/Emulsigen®D/IM		
Group 3	PBS/ Polyethyleneimine/IN	*S. suis* P1/7	3
	PBS/ Carbopol® & AddaVax™/IM		
Group 4	PBS/ Polyethyleneimine/IN	*S. suis* P1/7	3
	PBS/ Emulsigen®D/IM		
Group 5	PBS/none/IN	*S. suis* P1/7	4
	PBS/none/IM		

691 IN = intransal; IM = intramuscular; PBS = phosphate buffered saline

692

693

694 Table 5. Bacterial strains and vectors used in this study.

S. suis pig isolates	Serotype	Clinical association[a]	Tissue origin
P1/7	2	SYS-BRAIN	blood
SS021	1	SYS-OTHER	joint/skin
SS045	1	SYS-BRAIN	meninges
SS100	1/2	SYS-BRAIN	brain
SS043	1/2	RESP	lung
SS002	2	SYS-BRAIN	Brain
SS008	2	SYS-OTHER	pericardial swab
SS053	3	SYS-BRAIN	brain
SS084	3	RESP	lung
SS062	4	SYS-BRAIN	brain
SS079	4	SYS-BRAIN	brain
SS018	7	SYS-OTHER	Lung/pericardium
SS024	7	SYS-BRAIN	brain
SS068	8	SYS-BRAIN	brain
SS091	8	RESP-SD	lung
SS015	9	SYS-BRAIN	brain
SS088	9	SYS-OTHER	joint
SS078	10	SYS-OTHER	joint
SS063	14	SYS-OTHER	joint
SS077	14	SYS-BRAIN	brain
SS097	16	SYS-OTHER	spleen
SS037	22	RESP	lung
SS009	23	RESP	lung
SS082	31	RESP-SD	lung

E. coli strains and vector	Application
E. coli NovaBlue	*E. coli* host for cloning
E. coli BL21(DE3)	*E. coli* host for expressing recombinant protein
pET-30 Ek/LIC[b]	Vector for cloning, expression and purification of target proteins

695 [a] Isolates recovered from systemic sites in pigs with clinical signs and/or gross pathology
696 consistent with *S. suis* infection (including meningitis, septicaemia and arthritis) were classified
697 as systemic (SYS), whereas those recovered from the lung in the presence of gross lesions of
698 pneumonia were classified as respiratory (RESP). Isolates recovered from the lung of pigs with
699 pneumonia but also with gross signs of systemic streptococcal-type disease were classified as
700 RESP-SD.
701 [b] The pET-30 Ek/LIC vector is designed for cloning and high-level expression of target proteins
702 fused with the His•Tag® and S®Tag™ coding sequences that are cleavable with enterokinase
703 (Ek) protease. The plasmid contains a strong T7*lac* promoter, an optimized RBS, the coding
704 sequence for the Ek protease cleavage site (AspAspAspAspLys↓), and a multiple cloning site
705 that contains restriction enzyme sites found in many other Novagen expression vectors to
706 facilitate insert transfer. An optional C-terminal His•Tag coding sequence is compatible with
707 purification, detection, and quantification.
708

709 Table 6. Protein cloning primers used in this study.

Primer ID	Primer function	Sequence (5'-3')
0185-4F[a]	Cloning primers for SSU0185	GACGACGACAAGATGTTCCGTTTAGCAAAAGAAGAAC
0185-1167R		GAGGAGAAGCCCGGTTATTTTTCTAAAGGATGGATGA
1915-4F	Cloning primers for SSU1915	GACGACGACAAGATGAAACACAATCTCCTTAAGAGCG
1915-1257R		GAGGAGAAGCCCGGTTAGTTGCTGTGTTTTTGAGCAA
1215-82F	Cloning primers for SSU1215	GACGACGACAAGATGGGCTTTATTATTGGGAAAGG
1215-1831R		GAGGAGAAGCCCGGTTATTCTTTACTGGATTTTTTTC
1355-91F	Cloning primers for SSU1355	GACGACGACAAGATGTTAGCTGTCCAAATTATGGGAG
1355-2022R		GAGGAGAAGCCCGGTTACTCCCCTTCCTTACGTCTCA
1773-121F	Cloning primers for SSU1773	GACGACGACAAGATGGATACTAGTGGAGAAGGATTGG
1773-5076R		GAGGAGAAGCCCGGTTATTCTTTTCGCTTCAAATTTC

710 [a]Underlined nucleotides corresponded to the sequence extensions required for LIC compatibility

711 with the pET-30 Ek/LIC cloning vector.

712

713

714 Figure 1. SDS-PAGE and Western blots of the five candidate vaccine proteins. The five
715 candidate proteins were expressed in *E. coli* and purified as described in the Materials and
716 Methods. The purified proteins were run on SDS-PAGE (A) and also transferred to membranes
717 and probed with either serum from a pig experimentally infected with *S. suis* serotype 2 (B) or
718 sera from pigs raised in a pathogen free environment as a negative control (C).
719
720 Figure 2. IgG antibody titers among vaccinated pigs in groups 1 and 2 to the individual subunit
721 proteins on day 14 (2 weeks after priming) and days 21 and 28 (1 and 2 weeks after boost). Pigs
722 in Groups 1 and 2 (6 pigs each) were vaccinated with the 5 candidate proteins on days 0 and 14
723 of the experiment. Both groups were given the 5 proteins intranasally with polyethyleneimine as
724 adjuvant, in addition Group 1 pigs were given the 5 proteins intramuscularly with Addavax™
725 and Carbopol® as adjuvant, while Group 2 pigs were given the 5 proteins intramuscularly with
726 Emulsigen®-D as adjuvant. Titers were determined via indirect ELISA with plates coated with
727 the individual proteins using two-fold serial dilutions of serum. The resulting OD data were
728 modeled as a nonlinear function of the Log_{10} dilution using log (agonist) vs. response-variable
729 slope four-parameter logistic model. Endpoints were interpolated by using 2X the average OD
730 of the day 0 sample for each respective pig as the cutoff.
731
732 Figure 3. ELISpot data showing the number of IFN-γ secreting cells detected in PBMCs isolated
733 from pigs in the indicated groups on days 14 (2 weeks after priming), 21, and 28 (1 and 2 weeks
734 after boost). Pigs in Groups 1 and 2 (6 pigs each) were vaccinated with the 5 candidate proteins
735 on days 0 and 14 of the experiment. Both groups were given the 5 proteins intranasally with
736 polyethyleneimine as adjuvant, in addition Group 1 pigs were given the 5 proteins

intramuscularly with Addavax™ and Carbopol® as adjuvant, while Group 2 pigs were given the 5 proteins intramuscularly with Emulsigen®-D as adjuvant. Groups 3-5 were control groups given the adjuvants (Groups 3 and 4, 3 pigs each) alone or PBS (Group 5, 4 pigs). PBMC collected on days 14, 21 and 28 were seeded at $2.5x10^5$ cells per well in duplicate and stimulated with a protein pool of the 5 candidate proteins. Control wells were stimulated with media alone or pokeweed mitogen (data not shown). The treatment group means and standard errors of the means are denoted. Statistically significant differences between groups are identified by an asterisk ($P<0.05$).

Figure 4. Cytokines produced by PBMCs isolated from pigs in the indicated groups on day 28 (2 weeks after boost). Pigs in Groups 1 and 2 (6 pigs each) were vaccinated with the 5 candidate proteins on days 0 and 14 of the experiment. Both groups were given the 5 proteins intranasally with polyethyleneimine as adjuvant, in addition Group 1 pigs were given the 5 proteins intramuscularly with Addavax™ and Carbopol® as adjuvant, while Group 2 pigs were given the 5 proteins intramuscularly with Emulsigen®-D as adjuvant. Groups 3-5 were control groups given the adjuvants (Groups 3 and 4, 3 pigs each) alone or PBS (Group 5, 4 pigs). PBMCs collected on day 28 were stimulated *in vitro* with a pool of the 5 candidate proteins and the supernatants collected to evaluate cytokine levels secreted by the cells by multiplex cytokine ELISA. Data presented as box and dot plots with the mean cytokine concentration (pg/ml). Significantly different cytokine concentrations among groups are identified with different lettered superscripts ($P<0.05$).

Figure 5. Survival rates of pigs vaccinated with 5 subunit proteins with different adjuvant formulations (Groups 1 and 2) compared to pigs given adjuvant alone (Groups 3 and 4) or PBS (Group 5). Pigs in Groups 1 and 2 (6 pigs each) were vaccinated with the 5 candidate proteins on days 0 and 14 of the experiment. Both groups were given the 5 proteins intranasally with polyethyleneimine as adjuvant, in addition Group 1 pigs were given the 5 proteins intramuscularly with Addavax™ and Carbopol® as adjuvant, while Group 2 pigs were given the 5 proteins intramuscularly with Emulsigen®-D as adjuvant. Groups 3-5 were control groups given the adjuvants alone (Groups 3 and 4, 3 pigs each) or PBS (Group 5, 4 pigs).

Figure 6. Cross reactive IgG antibody to whole *S. suis* bacteria of serotypes that commonly cause systemic disease from Group 1 and 2 pigs on day 28 (2 weeks after boost). Pigs in groups 1 and 2 (6 pigs each) were vaccinated with the 5 candidate proteins on days 0 and 14 of the experiment. Both groups were given the 5 proteins intranasally with Polyethyleneimine as adjuvant, in addition group 1 pigs were given the 5 proteins intramuscularly with Addavax™ and Carbopol® as adjuvant, while group 2 pigs were given the 5 proteins intramuscularly with Emulsigen®-D as adjuvant. IgG reactivity was determined via indirect ELISA with plates coated with heat inactivated whole bacteria. Serum samples collected on day 28 from each pig were diluted 1:500 and used in the assay. Data is reported as the mean ± SEM optical density at 405 nm. Bacterial strains are listed on the X-axis with serotype in parentheses.

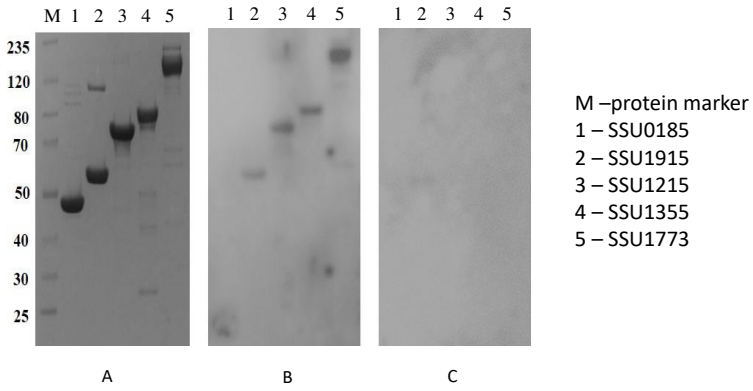

Figure 1

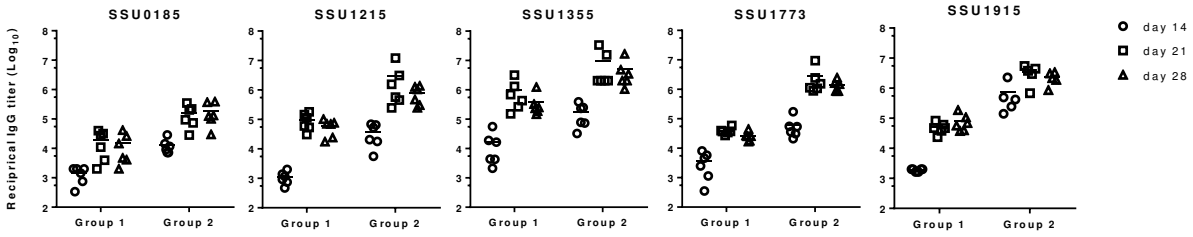

Figure 2

Figure 3

Figure 4

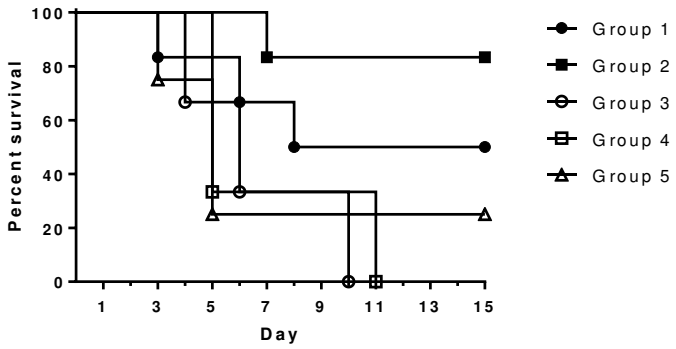

Figure 5

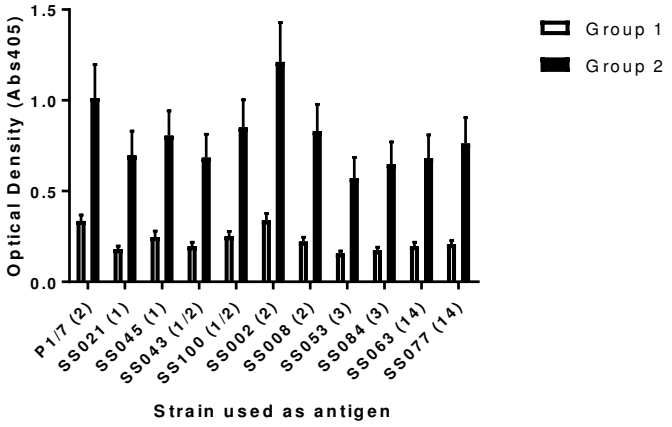

Figure 6