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

3

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

17

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23

24 **Abstract**

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

39

40 **Introduction**

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

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

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

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

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

77

78 **Results**

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

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

101 **Parenteral adjuvant formulation and boosting significantly impacts the serum IgG**
102 ***S. suis* protein specific response.** Two groups of pigs were vaccinated with the five proteins
103 both intranasally with Polyethyleneimine as adjuvant and intramuscularly with one of two
104 adjuvant combinations, AddaVax/Carbopol (group 1) or Emulsigen-D (group 2) as described in
105 the Materials and Methods section (Table 4). Groups 3-5 were control groups given PBS mixed
106 with the same adjuvants given to groups 1 and 2 or PBS only, respectively. Overall, serum IgG
107 antibody reactive against all five proteins was detected in all vaccinated pigs, and there was an
108 anamnestic response after administration of the boost vaccination (Figure 2). No *S. suis* protein-
109 specific IgG was detected in the pigs given adjuvant alone or PBS (data not shown), nor was
110 there a response detected in serum collected at day 0. Two weeks following priming (day 14),
111 IgG titers specific to individual *S. suis* proteins were significantly higher in serum from pigs in
112 group 2 (Emulsigen-D adjuvant) compared to group 1 (Carbopol/AddaVax adjuvant) and this
113 trend continued after the response was boosted (day 21 and 28). In fact, IgG titers to the proteins
114 in group 2 pigs after a single injection were approximately equal to the titers in group 1 pigs after
115 2 injections.

116 **Peripheral *S. suis* protein-specific IFN- γ recall response declines following boost**
117 **immunization.** The number of PBMCs producing IFN- γ following re-exposure to the pool of *S.*
118 *suis* proteins was used as a measure of vaccine-induced cell-mediated immunity. The number of
119 IFN- γ secreting cells (SC) following re-stimulation with *S. suis* proteins was greatest on day 14
120 post-priming, and adjuvant formulation had a significant impact on responses with pigs in group 1
121 2 (Emulsigen-D adjuvant) having significantly higher numbers of IFN- γ SC compared to group 1
122 (Carbopol/AddaVax adjuvant) (Figure 3). The number of IFN- γ SC detected decreased over
123 time; with an average of 263 and 32 IFN- γ SC for group 2 detected on days 14 and 28,
124 respectively. PBMC collected from pigs in groups 3, 4 and 5 (no antigen groups) did not have
125 more than 13 IFN- γ SC detected at any time point following stimulation *S. suis* proteins. In
126 addition, the number of IFN- γ SC detected following stimulation with media alone remained
127 below 10 at each time point evaluated. While there was, on average, an increase in the number of
128 IFN- γ SC using PBMC from pigs in group 1 at day 14 post-priming, it was not significantly
129 increased over control groups (groups 3-5).

130 **Cytokines produced by PBMCs following restimulation with the protein pool were**
131 **highest in pigs vaccinated with Emulsigen-D adjuvant.** PBMCs collected on day 28, 2 weeks
132 after boost vaccination, were stimulated with the pool of five *S. suis* proteins as another measure
133 of vaccine-induced cell-mediated immunity. Overall, cytokines produced by PBMCs following
134 restimulation with the protein pool were highest in pigs from group 2 (Emulsigen-D adjuvant)
135 (Figure 4). These levels were statistically higher for group 2 compared to all other groups for IL-
136 2 and TNF- α , whereas there was no statistical difference in the amount of these cytokines
137 produced among groups 1 (Carbopol/AddaVax adjuvant) and 3-5 (control groups).

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

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

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

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

175

176 **Discussion**

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

183 SSU0185 was identified as a putative tagatose-6-phosphate aldose/ketose isomerase. The
184 ortholog of this protein, AgaS, is believed to be part of the pathway for utilization of the amino
185 sugar, N-acetyl-D-galactosamine in *E. coli* (18). The abundance of free sugars is scarce in the
186 respiratory tract and mucins, a major component of the mucus produced by respiratory surfaces,
187 contain glycoproteins composed of sugars, amino sugars, and sulphated sugars commonly linked
188 to a protein core via an N-acetylgalactosamine (19). Orthologs of *agaS* have been identified in
189 other *Streptococcus* species, such as *Streptococcus pneumoniae*, where it was shown to be
190 upregulated upon exposure to human macrophage-like cells and when grown in the presence of
191 mucin, potentially explaining the importance of this protein for survival in the respiratory tract
192 (20, 21).

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

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

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

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

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

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

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

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

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

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

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

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

297

298 **Materials and methods**

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

307 *S. suis* (P1/7), a serotype 2 isolate from the blood of a pig with meningitis (40), was used
308 for challenge and was grown on tryptic soy agar containing 5% sheep blood (Becton, Dickinson
309 and Co.) at 37 °C overnight, scraped from the plates and resuspended in phosphate buffered
310 saline (PBS) to an optical density of 0.42 at A₆₀₀ to give an inoculum dose of 1 x 10⁹ cfu/ml.
311 Each challenged pig received 1 ml per nostril (2 ml total).

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

318 The polymerase chain reactions (PCRs) for screening bacterial colonies were set up with
319 Go Taq Green Master Mix (Promega Ltd.) according to the manufacturer's instructions. The
320 amplification conditions used were as follows: initial denaturation at 95 °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.

343 **Selection of candidate vaccine proteins.** A strategy of combining experimental
344 functional genomics screening (IVOC with TraDIS) with *in silico* bioinformatics approaches was
345 applied for selection of candidate vaccine proteins using a library generated in *S. suis* strain P1/7
346 (13-17, 41). The selection consists of the following steps: (1) candidate fitness genes (defined as
347 a gene that harbored at least one transposon insertion mutant with significant reduction in fitness
348 in a swine respiratory epithelium IVOC system) were determined through previous functional
349 genomics screening, (2) protein subcellular localization was predicted *in silico* with
350 bioinformatics approaches using PSORTb (<http://db.psort.org/>) and LocateP
351 (<http://www.cmbi.ru.nl/locatep-db/cgi-bin/locatepdb.py>) databases or based on literature
352 mining to shortlist fitness genes encoding surface-associated proteins [(cell wall anchored or
353 extracellular (lipid-anchored or secretory)], (3) proteins containing transmembrane domains in
354 the middle of protein coding sequence were excluded, (4) *in silico* protein homology based
355 searches to identify proteins with cross-protection potential: i.e. the presence of the protein from
356 the *S. suis* P1/7 genome was used as a query in a BlastX search and we identified proteins
357 present (80% identity over 80% of the length) in 459 publically available strains or in the
358 majority of disease-associated strains (12), (5) a final pool with five potential candidate vaccine
359 proteins were chosen whose potential to be applied as a cassette of subunit vaccine has not been
360 previously published, patented or tested in pig protection studies.

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

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

373 **Purification of recombinant vaccine proteins.** Recombinant proteins were purified
374 from 1-6 L cultures grown in 2YT broth and induced with 1 mM IPTG for 2 to 4 hours. Cell
375 pellets were washed once in PBS and centrifuged at $3,000 \times g$ for 15 minutes. The cell pellets
376 were resuspended in binding buffer (10 mM imidazole, 300 mM NaCl, 50 mM phosphate,
377 pH:8.0) and sonicated on ice for 6 minutes. Appropriate amounts of Benzonase and rLysozyme
378 (Novagen, Merck Millipore) were added to reduce the viscosity of the lysate and improve protein
379 extraction efficiency. The lysates were first centrifuged at $3,000 \times g$ for 10 minutes at 4 °C to
380 pellet debris and the supernatants were subjected to further centrifugation at $75,000 \times g$ for 1.5
381 hours at 4 °C. Recombinant proteins were subjected to purifications by nickel His-Tag affinity
382 chromatography, anion exchange chromatography, CHAP chromatography and gel filtration
383 when appropriate. Target proteins were confirmed by peptide mass fingerprinting. Protein
384 concentration was determined using spectrophotometry and purified proteins were stored at -80
385 °C.

386 **Immunoreactivity of the recombinant proteins with convalescent pig sera.**

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

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

403 **Vaccine protection study.** The USDA-ARS-National Animal Disease Institutional
404 Animal Care and Use Committee approved all animal work. Twenty-two, 5-week-old,
405 Caesarean-derived, colostrum-deprived (CDCD) pigs were distributed into groups as follows
406 (Table 3): group 1 pigs (6 pigs) were given a 2 ml dose of vaccine containing 250 µg protein (50
407 µg per subunit) with 1ml of Addavax™ emulsion (Squalene-based oil-in-water adjuvant-
408 Invivogen), and 5 mg of Carbopol®-971 (Lubrizol Corporation) intramuscularly (IM) in the neck
409 and a 2 ml dose of vaccine containing 500 µg protein (100 µg per subunit) and 500 µg of
410 Polyethyleneimine (Sigma) intranasally (IN- 1 ml per nostril); group 2 pigs (6 pigs) were
411 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 $-80\text{ }^{\circ}\text{C}$
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

435 buffer (pH 9.6) overnight at 4 °C at the following concentrations: SSU1773 (1 µg/ml), SSU1355
436 (2 µg/ml), SSU1915 (1 µg/ml), SSU0185 (1 µg/ml), SSU1215 (0.5 µg/ml). The next day, plates
437 were blocked with 0.2 ml of blocking buffer [2% BSA in PBS tween (0.05% Tween-20; PBS-T)]
438 for 2 hours at RT and then washed three times with PBS-T. Eleven, two-fold serial dilutions of
439 serum (starting at 1:2000) collected from each pig were made in 1% BSA/PBS-T, transferred to
440 the ELISA plate in duplicate and incubated at RT for 2 hours. Plates were washed and *S. suis*
441 specific IgG detected by adding 0.1 ml of anti-porcine IgG conjugated to horseradish peroxidase
442 (KPL, catalog 14-14-06, dilution 1:10,000) and incubating at RT for 1 hour. Plates were washed
443 and TMB substrate added according to manufacturer's recommendations (Life Technologies).
444 After 15 minutes with substrate, 0.05 ml of stop solution (2N H₂SO₄) was added and optical
445 density read at 450 nm with correction at 655 nm. The resulting OD data were modeled as a
446 nonlinear function of the Log₁₀ dilution using Graph Pad Prism (La Jolla, CA) log (agonist) vs.
447 response-variable slope four-parameter logistic model. Endpoints were interpolated by using 4X
448 the average OD of the day 0 sample of each respective pig serum as the cutoff.

449 To determine whether serum IgG reacted with whole P1/7 *S. suis* bacteria, heat-
450 inactivated (HI) P1/7 was used as antigen in an indirect ELISA. To make antigen, a single P1/7
451 colony was inoculated into 5 ml THB and incubated at 37 °C in 5% CO₂ at 200 rpm for
452 approximately 6 hours, at which time it had reached an OD=0.6 at Abs600. The bacteria were
453 centrifuged at 4000 x g to pellet, media decanted and bacteria resuspended in 5 ml PBS. Bacteria
454 were heat-inactivated (HI) by incubating the suspension in a water bath at 85 °C for 20 minutes.
455 Inactivation was confirmed by plating 0.1 ml of the heat-inactivated preparation on blood agar
456 plates and incubating the plates at 37 °C in 5% CO₂. No growth was observed on the plate after 2
457 days. Aliquots were stored frozen at -80 °C. Protein concentration of the HI P1/7 was determined

458 using BCA protein microtiter assay according to manufacturer's recommendations (Pierce).
459 Immulon-2 plates were coated with 0.1 ml of 7.5 µg/ml of HI P1/7 diluted in 100 mM carbonate-
460 bicarbonate buffer (pH 9.6). Serum samples collected on day 0 and day 28 from each pig were
461 diluted 1:500 and used in the assay. P1/7-specific IgG was detected and the ELISA completed as
462 described above for individual proteins. Data is reported as the OD at 450 nm with correction at
463 655 nm. A checkerboard of HI P1/7 concentrations and a pool of sera from day 0 and day 28 was
464 used to determine optimal ELISA conditions (data not shown). Similar techniques were used to
465 evaluate IgG reactivity with a collection of other HI *S. suis* strains comprised of two randomly
466 selected representatives of those serotypes most commonly associated with disease (1, 2, 1/2, 3
467 and 14) (see Table 1), with bacteria reaching OD's of 0.6 - 1.1 at 600 nm in the 6-8 hour culture
468 period prior to HI (data not shown) and all HI *S. suis* coated at 7.5 µg/ml for the ELISA.

469 **Evaluation of the cell-mediated immune response to vaccination.** To evaluate
470 induction of cell-mediated immunity following vaccination, ELISpot assays were performed to
471 enumerate IFN-γ-secreting cells following *in vitro* stimulation with a pool of the vaccine
472 proteins. Blood was collected by venipuncture into a BD Vacutainer Cell Preparation Tubes
473 (CPT) with sodium citrate for isolation of PBMC using culture media as previously described
474 (42). PBMC were enumerated and seeded at 2.5×10^5 cells per well in the IFN-γ ELISpot plates in
475 duplicate for each treatment. PBMC were stimulated with a protein pool in final volume of 0.25
476 ml (1 µg/ml of each individual protein per well). Control wells received media alone or
477 pokeweed mitogen (0.5 µg/ml). Approximately 18 hours after stimulation the ELISpot assay was
478 completed according to manufacturer's recommendations (R&D Systems, Minneapolis, MN).
479 Spots were enumerated using a S5UV ImmunoSpot instrumentation and software (Cellular
480 Technology Ltd., Shaker Heights, OH) and data analyzed using GraphPad Prism software (La

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

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

490 **Statistical Analysis.** Survival analysis was performed using the product limit method of
491 Kaplan and Meier, and comparing survival curves using the logrank test (GraphPad Prism, La
492 Jolla, CA). Antibody titers were Log10 converted and a two-tailed student's t-test was used to
493 evaluate statistical differences between groups 1 and 2 for indicated comparisons, with a p-value
494 <0.05 considered significant. One-way analysis of variance (ANOVA) with a Tukey's multiple
495 comparison post-test was performed to evaluate statistical differences between groups (p<0.05)
496 for the number of IFN- γ secreting cells and cytokine production. Graph Prism software (version
497 6.0) was used for statistical analysis.

498

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661 Table 1. Characteristics of the five candidate vaccine proteins

Antigen encoding genes	Function/ortholog	Range of Full length		N- terminal signal peptide ^b (AA)	Protein subcellular localization prediction ^c	Conserved Domain	Fusion protein ^d AA / KDa
		TraDIS fitness scores ^a	protein residues (AA)				
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 (<i>in silico</i>)	LPSTG	623 / 67.4
SSU1355	Putative surface-anchored 5'-nucleotidase	-0.81 to 8.23 (3/4)	674	1-30	Cell-wall anchored (<i>in silico</i>)	LPNTG	687 / 74.1
SSU1773	Putative surface-anchored serine protease	1.00 to 8.74 (6/8)	1692	1-40	Cell-wall anchored (<i>in silico</i>)	LPQTG	1695 / 187.4
SSU1915	Putative maltose/maltodextrin-binding protein precursor (MalX)	-5.03 to 5.05 (2/2)	419	/	Lipid-anchored (<i>in silico</i>)	/	462 / 49.0

662 ^aTraDIS fitness scores were presented as log₂ 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 <i>S. suis</i> 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 ^aThe 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 ^bIsolates 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 ^aThe panel contains 2 representatives (where possible) of disease associated serotypes.

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

687 ^bStrains in bold also used in cross reactive ELISAs shown in Figure 6.

688 ^cNP = 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	<i>S. suis</i> proteins/Polyethyleneimine/IN	<i>S. suis</i> P1/7	6
	<i>S. suis</i> proteins/Carbopol® & AddaVax™/IM		
Group 2	<i>S. suis</i> proteins/ Polyethyleneimine/IN	<i>S. suis</i> P1/7	6
	<i>S. suis</i> proteins/Emulsigen®D/IM		
Group 3	PBS/ Polyethyleneimine/IN	<i>S. suis</i> P1/7	3
	PBS/ Carbopol® & AddaVax™/IM		
Group 4	PBS/ Polyethyleneimine/IN	<i>S. suis</i> P1/7	3
	PBS/ Emulsigen®D/IM		
Group 5	PBS/none/IN	<i>S. suis</i> P1/7	4
	PBS/none/IM		

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

692

693

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

<i>S. suis</i> 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
<i>E. coli</i> strains and vector	Application		
<i>E. coli</i> NovaBlue	<i>E. coli</i> host for cloning		
<i>E. coli</i> BL21(DE3)	<i>E. coli</i> 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 *T7lac* 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	<u>GACGACGACAAGATGTTCCGTTTAGCAAAAGAAGAAC</u>
0185-1167R		<u>GAGGAGAAGCCCGGTTATTTTTCTAAAGGATGGATGA</u>
1915-4F	Cloning primers for SSU1915	<u>GACGACGACAAGATGAAACACAATCTCCTTAAGAGCG</u>
1915-1257R		<u>GAGGAGAAGCCCGGTTAGTTGCTGTGTTTTTGAGCAA</u>
1215-82F	Cloning primers for SSU1215	<u>GACGACGACAAGATGGGCTTATTATTGGGAAAGG</u>
1215-1831R		<u>GAGGAGAAGCCCGGTTATTCTTACTGGATTTTTTTC</u>
1355-91F	Cloning primers for SSU1355	<u>GACGACGACAAGATGTTAGCTGTCCAAATTATGGGAG</u>
1355-2022R		<u>GAGGAGAAGCCCGGTTACTCCCCTCCTTACGTCTCA</u>
1773-121F	Cloning primers for SSU1773	<u>GACGACGACAAGATGGATACTAGTGGAGAAGGATTGG</u>
1773-5076R		<u>GAGGAGAAGCCCGGTTATTCTTTTCGCTTCAAATTC</u>

710 ^aUnderlined 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 AddavaxTM
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 Log₁₀ 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

737 intramuscularly with Addavax™ and Carbopol® as adjuvant, while Group 2 pigs were given the
738 5 proteins intramuscularly with Emulsigen®-D as adjuvant. Groups 3-5 were control groups
739 given the adjuvants (Groups 3 and 4, 3 pigs each) alone or PBS (Group 5, 4 pigs). PBMC
740 collected on days 14, 21 and 28 were seeded at 2.5×10^5 cells per well in duplicate and stimulated
741 with a protein pool of the 5 candidate proteins. Control wells were stimulated with media alone
742 or pokeweed mitogen (data not shown). The treatment group means and standard errors of the
743 means are denoted. Statistically significant differences between groups are identified by an
744 asterisk ($P < 0.05$).

745

746 Figure 4. Cytokines produced by PBMCs isolated from pigs in the indicated groups on day 28 (2
747 weeks after boost). Pigs in Groups 1 and 2 (6 pigs each) were vaccinated with the 5 candidate
748 proteins on days 0 and 14 of the experiment. Both groups were given the 5 proteins intranasally
749 with polyethyleneimine as adjuvant, in addition Group 1 pigs were given the 5 proteins
750 intramuscularly with Addavax™ and Carbopol® as adjuvant, while Group 2 pigs were given the
751 5 proteins intramuscularly with Emulsigen®-D as adjuvant. Groups 3-5 were control groups
752 given the adjuvants (Groups 3 and 4, 3 pigs each) alone or PBS (Group 5, 4 pigs). PBMCs
753 collected on day 28 were stimulated *in vitro* with a pool of the 5 candidate proteins and the
754 supernatants collected to evaluate cytokine levels secreted by the cells by multiplex cytokine
755 ELISA. Data presented as box and dot plots with the mean cytokine concentration (pg/ml).
756 Significantly different cytokine concentrations among groups are identified with different
757 lettered superscripts ($P < 0.05$).

758

759 Figure 5. Survival rates of pigs vaccinated with 5 subunit proteins with different adjuvant
760 formulations (Groups 1 and 2) compared to pigs given adjuvant alone (Groups 3 and 4) or PBS
761 (Group 5). Pigs in Groups 1 and 2 (6 pigs each) were vaccinated with the 5 candidate proteins on
762 days 0 and 14 of the experiment. Both groups were given the 5 proteins intranasally with
763 polyethyleneimine as adjuvant, in addition Group 1 pigs were given the 5 proteins
764 intramuscularly with AddavaxTM and Carbopol[®] as adjuvant, while Group 2 pigs were given the
765 5 proteins intramuscularly with Emulsigen[®]-D as adjuvant. Groups 3-5 were control groups
766 given the adjuvants alone (Groups 3 and 4, 3 pigs each) or PBS (Group 5, 4 pigs).

767

768 Figure 6. Cross reactive IgG antibody to whole *S. suis* bacteria of serotypes that commonly
769 cause systemic disease from Group 1 and 2 pigs on day 28 (2 weeks after boost). Pigs in groups
770 1 and 2 (6 pigs each) were vaccinated with the 5 candidate proteins on days 0 and 14 of the
771 experiment. Both groups were given the 5 proteins intranasally with Polyethyleneimine as
772 adjuvant, in addition group 1 pigs were given the 5 proteins intramuscularly with AddavaxTM and
773 Carbopol[®] as adjuvant, while group 2 pigs were given the 5 proteins intramuscularly with
774 Emulsigen[®]-D as adjuvant. IgG reactivity was determined via indirect ELISA with plates coated
775 with heat inactivated whole bacteria. Serum samples collected on day 28 from each pig were
776 diluted 1:500 and used in the assay. Data is reported as the mean \pm SEM optical density at 405
777 nm. Bacterial strains are listed on the X-axis with serotype in parentheses.

778

779

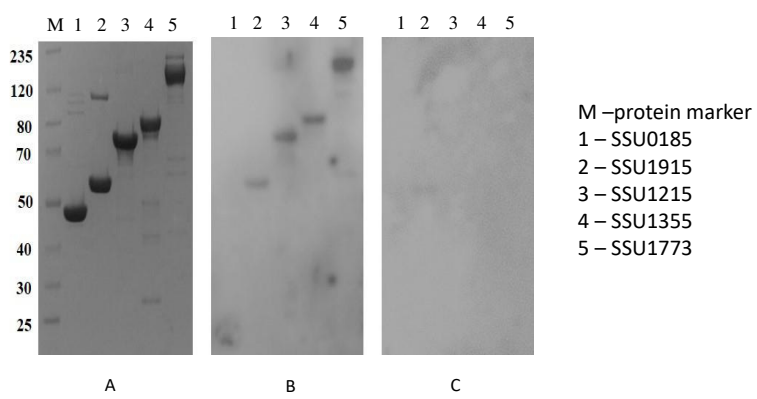


Figure 1

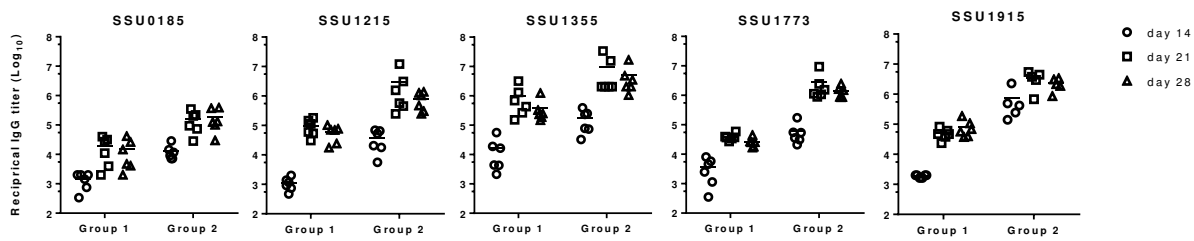


Figure 2

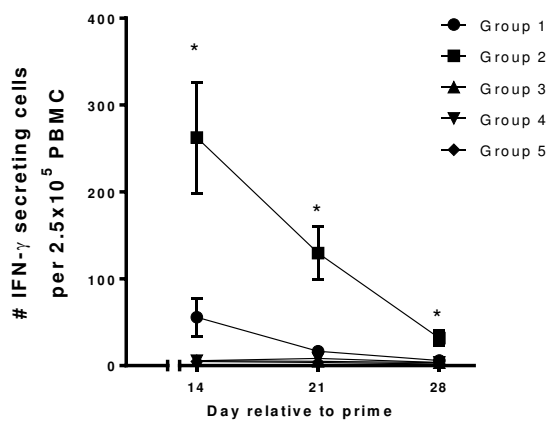


Figure 3

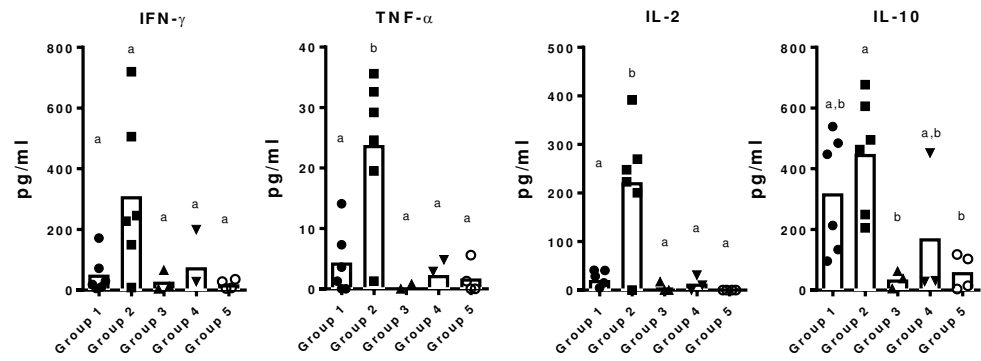


Figure 4

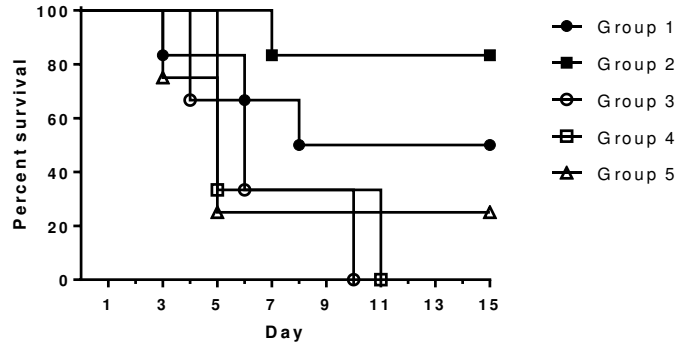


Figure 5

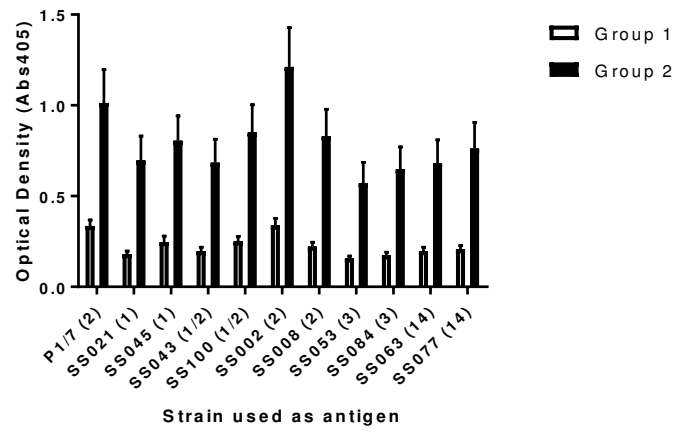


Figure 6