

Immunoproteomic assay of secreted proteins of *Streptococcus suis* serotype 9 with convalescent sera from pigs

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Abstract *Streptococcus suis* is an important pathogen of pigs. In China, in addition to *S. suis* serotype 2, *S. suis* serotype 9 (SS9) is also a prevalent serotype. There is no vaccine available for SS9. An immunoproteome-based approach was developed to identify SS9 immunogenic proteins for vaccine development. Secreted proteins extracted from SS9 strain GZ0565 were screened by two-dimensional Western blotting using convalescent sera from pigs. Protein spots were excised from preparative gels and were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, which led to the identification of ten immunogenic proteins (sortases, ABC transporter substrate-binding protein–maltose/maltodextrin, ABC transporter periplasmic protein, CHAP domain containing protein, peptidoglycan-binding LysM, elongation factor Tu, elongation factor G, thymidine kinase, molecular chaperone DnaK, hypothetical protein SSU98_2184). These novel immunogenic proteins, which are encoded by genes that are reasonably conserved among SS9 strains, may be developed as antigens for further study of SS9 vaccine.

Introduction

Streptococcus suis, a Gram-positive bacterium, is an important pathogen of pigs and is associated with numerous diseases, such as, meningitis, arthritis, bronchopneumonia, and septicemia (Gottschalk and Segura 2000). Among 33 serotypes, *S. suis* serotype 2 (SS2) is the most widely

isolated serotype. In China, in addition to SS2, *S. suis* serotype 9 is another prevalent serotype, which is frequently isolated from diseased pigs (Yu et al. 2007; Wu et al. 2008a, b). Unlike SS2, little attention has been given for development of vaccine specific to SS9. Secreted proteins play an important role in bacterial pathogenesis, and they are also involved in colonization, adhesion to and invasion of host cells. It was observed to be feasible to identify secreted proteins as potential vaccine candidates. Immunoproteomics, a technique involving 2-DE in combination with Western blotting has been successfully applied in the discovery of antigens from secreted proteins in SS2 (Zhang and Lu 2007b), *Shigella flexneri* (Ying et al. 2005), *Staphylococcus aureus* (Ziebandt et al. 2001).

In our previous study, eight surface SS9 immunogenic proteins were identified by immunoproteomic analysis with anti-SS9 sera from immunized mice (Wu et al. 2008b). However, it would be more informative if the sera were collected from pigs especially considering the development of vaccines for pigs. In this study, new SS9 immunogenic secreted proteins were identified by immunoproteomic analysis with convalescent sera pooled from pigs, which could contribute to the vaccine development for SS9.

Materials and methods

Bacteria strains and culture conditions

The SS9 strains used in this study are listed in Table 1. The SS9 strain GZ0565 was isolated from the organs of diseased pigs (Wu et al. 2008b; Yu et al. 2007). The SS9 strain GZ0565 were grown in Todd-Hewitt broth (Oxoid) and on Columbia agar blood base (Oxoid) containing 6% (v/v) sheep blood at 37°C.

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Table 1 Origins and the relevant characteristics of the SS9 strains

Strains	Relevant characteristic	Source
GZ0565	Clinical isolate	Guangzhou, China, 2005
GD0606	Clinical isolate	Guangdong, China, 2007
SH0626	Clinical isolate	Shanghai, China, 2007
SH0627	Slaughter house isolate	Shanghai, China, 2007
SH040917	Slaughter house isolate	Shanghai, China, 2004
SH89	Clinical isolate	Shanghai, China, 2006
JX041226	Slaughter house isolate	Jiangxi, China, 2004
NJ0607	Clinical isolate	Nanjing, China, 2006
SZ0608	Slaughter house isolate	Suzhou, China, 2006
CZ0608	Slaughter house isolate	Changzhou, China, 2006
YC0609	Clinical isolate	Yancheng, China, 2006
TZ0510	Clinical isolate	Taizhou, China, 2005
XZ0503	Clinical isolate	Xuzhou, China, 2005
22083	Reference strain	Denmark

Preparation of convalescent sera

Three 3–4-month-old Guangdong pigs from a herd that was free of *S. suis* were inoculated with SS9 strain GZ0565. The bacteria were cultured to late log phase, harvested by centrifugation at $10,000\times g$ for 10 min, then rinsed and diluted with sterile phosphate-buffered saline (pH 7.4). The pigs were challenged intravenously at a dose of 5×10^6 CFU per pig. All three pigs developed severe signs of acute arthritis and showed fever (40–40.5°C), anorexia, kyphosis, ataxia, and tremor. All the three pigs survived. Blood samples were collected from pigs prior to inoculation, 1 month after the inoculation. Sera from pre-inoculation and convalescent pigs were pooled for immunological assays.

Preparation of secreted proteins

Preparations of secreted proteins were performed as described in our previous study (Wu et al. 2008a). Briefly, culture supernatant of strain GZ0565 was harvested at late log phase by centrifugation ($10,000\times g$ for 15 min at 4°C). The residual bacteria in the supernatants were removed through 0.22 μ m filter membrane. Trichloroacetic acid was added to the filtrate to a final concentration of 10%, and then the filtrate was incubated in ice water for 30 min. After centrifugation for 15 min at $10,000\times g$ and 4°C, the pellet was washed with prechilled acetone two times. The pellet was air-dried and was stored at –70°C.

Two-dimensional gel electrophoresis

Secreted proteins were treated with 2-D Clean-up Kit (GE Healthcare) before rehydration. Isoelectric focusing

(IEF) was performed in a 130-mm Immobiline DryStrip with a pH 4–7 gradient using Ettan IPGphorIII (GE Healthcare). Immobiline strip rehydration was performed overnight in 250 μ L rehydration/sample buffer (8 mol/L urea, 2% CHAPS, 50 mmol/L DTT, 0.2% Bio-Lyte, 4–7 Pharmalyte, 0.001% bromphenol blue; Bio-Rad) containing 300- μ g protein samples. IEF was performed in five steps: at 500 V for 4 h, at 1,000 V for 1 h, at 2,000 V for 1 h, at 4,000 V for 1 h, and at 8,000 V for 2.5 h.

The IPG strips were equilibrated for 15 min each in 3 mL of solution 1 (75 mmol/L Tris–HCl (pH 8.8), 6 mol/L urea, 29.3% glycerin, 2% SDS, 1% DTT) and then in 3 mL of solution 2 (5 mmol/L Tris–HCl (pH 8.8), 6 mol/L urea, 29.3% glycerin, 2% SDS, 2.5% iodacetamide). The proteins were separated in 12% polyacrylamide gel without a spacer gel. Gels were run at 15 mA per gel for 30 min and then at 30 mA per gel for separation until the dye reached the bottom of the gels. To visualize the separated proteins, the gels were stained with colloidal Coomassie Blue G-250 (CBB) and then analyzed by the software PDQuest v 7.3 (Bio-Rad). Three replicates were run for each sample.

Western blotting

Another gel run in parallel was used for Western blotting. Secreted proteins from gels were transferred to a polyvinylidene fluoride membranes (GE Healthcare) for 2 h at 0.8 mA/cm² in transfer buffer (39 mmol/L glycine, 48 mmol/L Tris, 20% methanol, 0.037% SDS) by using a semidry blotting apparatus TE77 (GE Healthcare). The membrane was blocked for 2 h at room temperature with blocking buffer PBST (5% skim milk in PBS buffer (pH 7.4) containing 0.05% Tween-20). The convalescent sera pooled from the pigs were used as an antibody source, and the pre-inoculation sera were used as a negative control. The membrane was incubated for 1 h at room temperature with the pooled sera (a dilution of 1:1,000). The membrane was then rinsed with PBST and incubated with Staphylococcal protein A-HRP (SPA-HRP, Boster; 1:10,000 dilution) at room temperature. After washing with PBST buffer, the membranes were treated with Super Signal West Pico Chemiluminescent Substrate (PIERCE) and then exposed to a Kodak film for 1 min. All experiments were done in triplicate.

Mass spectrometry analysis of protein spots and database searches

Protein spots were excised from stained 2-D gels and were sent to Shanghai GeneCore BioTechnologies Co. Ltd. (Gene Core Ltd., China) for tryptic in-gel digestion

and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). For MALDI-TOF-MS, the samples were analyzed in the positive ion, reflectron mode, on a TOF Ultraflex II mass spectrometer (Bruker Daltonics). Each acquired mass spectra (m/z range, 700–4,000; resolution, 15,000–20,000) was processed using the software FlexAnalysis v2.4 software (Bruker Daltonics) with the following settings: peak detection algorithm set at Sort Neaten Assign and Place, S/N threshold at 3, and quality factor threshold at 50. Search peptide mass fingerprinting (PMF) data in the online tool MASCOT server (<http://www.matrixscience.com>). Search parameters were one max missed cleavage, variable modification of oxidation (M), and peptide mass tolerance of 100 ppm. The criteria for the identification of proteins were PMF data, including sequence coverage, matching peptide masses and scores in MASCOT. Protein identification was assigned when the following criteria were met: at least four matching peptides and sequence coverage greater than 15% (Wu et al. 2008a).

PCR detection

Primers were synthesized according to the genes encoding the identified protein (Table 2). The program used consisted of an incubation for 5 min at 95°C and 30 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C, followed by an

incubation for 10 min at 72°C. Distribution of the genes in the SS9 genome was determined by PCR analysis of the SS9 strains listed in Table 1.

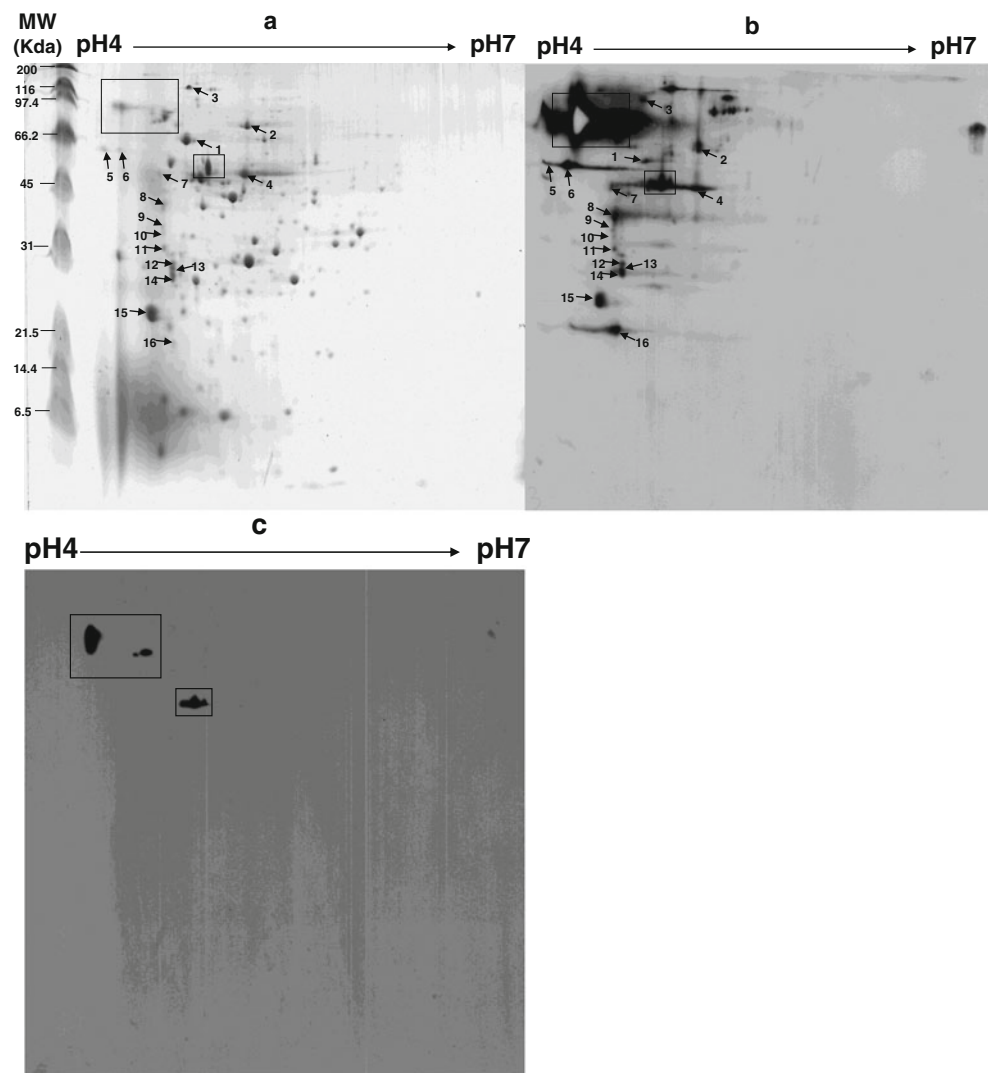
Results and discussion

Here, we focused on the analysis of secreted proteins from late log phase, with a pH range of 4–7, of SS9 strain GZ0565. Two lines of rationale dictated the strategy of the study: (1) bacteria in log growth are usually in their healthiest state, and thus, cells in the log phase are often desirable for studies of virulence or other cell components; (2) according to our preliminary studies, most of the protein spots were displayed within the pH range of 4–7. Thus, it is possible that some secreted proteins that are secreted after the log phase or some proteins that cannot be displayed within the pH range of 4–7 may be missed in our study. Secreted proteins of SS9 strain GZ0565 were resolved on a 2-DE gel. After 2-DE, CBB-stained gel revealed about 120 spots using a pH 4–7 130 mm IPG strip (Fig. 1a). Most of the proteins bore a molecular weight between 15 and 116 kDa. Sixteen protein spots with high repeatability were recognized by convalescent sera pooled from pigs (Fig. 1b). When the blot was probed with pre-inoculation sera, protein spots shown in oblong area were observed (Fig. 1c). Therefore, these protein spots shown in the oblong area should be removed from the results of

Table 2 Primers for genes encoding immunogenic proteins

Primers	Primer sequence (5'-3')	Detected gene of protein	GenBank accession number	gi number in NCBI	Length of PCR products (bp)
dna	GCAAAACGCCAAGCGGTAAC TGATGAATGGCAAGCTGATT	Molecular chaperone DnaK	NC_009442 REGION: 291200..293023	gi 146317956	613
efg	CGCGGGTAAAACAACGACAA ATAACCGCATCAAGCATCAACT	Translation elongation factor G	NZ_AAFA03000036 REGION: 50..2131	gi 223933803	765
eft	CGTCGCTTGCCTTCATCAGT TTTGTGTCAGTGTGCGTTCTG	Elongation factor Tu	NC_009442 REGION: 513622..514836	gi 189037416	525
sor	CCTAGCGTTGATATTCAACAC GACACTTTCAGGCGAAACAA	Sortase	AB081330 REGION: 2701..3450	gi 28189059	487
abc	TTAGCTGCATGCTCAAACCTC GAATACATAGCCGCCGTAAC	ABC transporter Substrate-binding Protein - maltose/ maltodextrin	NC_009442 REGION: 2036511..2037770	gi 146319787	570
bml	CTGCATGTGGTAACCGTGCT GAAACCAGCTTCAAAGCGAT	ABC transporter periplasmic protein	NZ_AAFA03000002 REGION: 89543..90610	gi 223932345	506
cha	AACAGGCGAAATTAACAGAAG TCCAATTCAGCCTGACGCAC	CHAP domain containing protein	NZ_AAFA03000002 REGION: 135266..136522	gi 223932390	388
tk	CCACAATATGAAGAACAAG ATGTAGGTTTCATTGCCACC	Thymidine kinase	NZ_AAFA03000001 REGION: 23755..24336	gi 223932121	462
lys	TAGCGGAATCTCCCTTTTA AAGCATCTGCTACACGCTCT	Peptidoglycan-binding LysM	NZ_AAFA03000029 REGION: 2693..3271	gi 223933683	438
hyp	TCATGGCCATTGGATTAAAC ACCAGACATCATGCGGTTGA	Hypothetical protein SSU98_2184	NC_009443 REGION: 2086225..2087097	gi 146322031	518

Fig. 1 2-DE proteome map (130 mm IPG strip, pH 4–7) and immunoblot analysis of secreted protein from SS9 strain GZ0565. **a** Preparative gel stained with CBB. Molecular weight markers are on the *left* in kilodalton. Another gel run in parallel was used for Western blotting using **b** convalescent sera pooled from pigs or **c** pre-inoculation sera pooled from pigs (all sera were diluted 1:1,000). Spots identified by MALDI-TOF-MS are labeled



immunoreactive proteins. These 16 protein spots were identified by MALDI-TOF-MS. The 11 successfully identified immunoreactive spots corresponded to ten individual proteins. The results are summarized in Table 3.

Spot 6 matched the *S. suis* sortases. Sortases, an enzyme involved in the covalent linkage of surface proteins to the peptidoglycan, are a family of Gram-positive bacterial transpeptidases (Race et al. 2009). They play critical roles in the virulence of Gram-positive bacterial pathogens such as SS2 (Wang et al. 2009), *Streptococcus agalactiae* (Lalioui et al. 2005), *Streptococcus pneumoniae* (Paterson and Mitchell 2006), and *Listeria monocytogenes* (Bierne et al. 2002). Wang et al. reported that the deletion of sortase A gene attenuated the full virulence of SS2 strain 05ZYH33 and impaired its colonizing potential in specific organs (Wang et al. 2009). Gianfaldoni et al. found that intraperitoneal immunization with recombinant sortase A conferred protection to mice against *S. pneumoniae* intraperitoneal challenge (Gianfaldoni et al. 2009). These findings suggested that sortases are attractive targets for vaccine development.

In pathogenic bacteria, ABC systems are known to play roles in virulence and pathogenicity because they are associated with many physiological processes (uptake of nutrients, non-classical secretion of signalling molecules, multidrug resistance) (Davidson and Chen 2004). Some of them are attractive targets for both vaccine and anti-infective development (Tanabe and Grenier 2009). Some of these proteins have been identified as immunogenic protein in *S. suis* (Zhang et al. 2008; Wu et al. 2008b). The protein from spot 7 proved to be *S. suis* ABC transporter substrate-binding protein–maltose/maltodextrin. It is involved in the utilization of maltose and maltodextrins. The uptake of nutrients is essential for the survival of bacterial cells. It was identified as immunogenic protein in SS2 by Zhang et al. (2008). Spot 8 was a match to *S. suis* ABC transporter periplasmic protein. The gene shows high homology to *S. suis* basic membrane lipoprotein. The antigenic membrane lipoprotein from *Actinobacillus pleuropneumoniae*, with high homology to ABC transporter periplasmic protein, was proved to be a highly immuno-

Table 3 Protein spots identified by MALDI-TOF-MS

Spot number	Identified protein		Theoretical MW (Da)/pI	Experimental MW (Da)/pI ^b	Mascot Score ^c	No. of peptides matched ^d	Coverage (%) ^e
	Mascot results ^a	Annotation/species					
1	gi 146317956	Molecular chaperone DnaK/ <i>S. Suis</i> 05ZYH33	64787/4.62	66000/4.8	111	19	37
2	gi 223933803	Translation elongation factor G/ <i>S. Suis</i> 89/1591	76677/4.89	75000/5.0	220	32	46
4	gi 189037416	Elongation factor Tu/ <i>S. Suis</i> 05ZYH33	44019/4.87	45000/5.1	221	31	72
6	gi 28189059	Sortase/ <i>S. Suis</i>	27893/5.52	60000/4.2	169	12	53
7	gi 146319787	ABC transporter substrate- binding protein - maltose/ maltodextrin/ <i>S. Suis</i> 05ZYH33	44245/4.63	48000/4.5	86	12	37
8	gi 146320941	ABC transporter periplasmic protein/ <i>S. Suis</i> 98HAH33	36292/4.67	38000/4.5	94	15	45
11	gi 223932390	CHAP domain containing protein/ <i>S. Suis</i> 89/1591	44542/4.97	31000/4.5	113	15	30
12	gi 223932390	CHAP domain containing protein/ <i>S. Suis</i> 89/1591	44542/4.97	30000/4.7	88	14	28
13	gi 223932121	Thymidine kinase/ <i>S. Suis</i> 89/1591	22105/5.58	26000/4.7	183	11	50
15	gi 223933683	Peptidoglycan-binding LysM/ <i>S. Suis</i> 89/1591	20843/4.84	23000/4.4	125	11	30
16	gi 146322031	Hypothetical protein SSU98_2184/ <i>S. Suis</i> 98HAH33	31634/5.93	20000/4.7	148	12	27

^a gi number in NCBI: gi number is simply a series of digits that are assigned consecutively to each sequence record processed by NCBI; protein sequence gi number is shown in the CDS/db_xref field of a nucleotide database record

^b Observed MW and pI of protein spot in the 2-D gel

^c Protein score greater than 82 is significant in this study ($p < 0.05$)

^d Number of peptides that match the predicted protein sequence

^e Percentage of predicted protein sequence covered by matched peptides

genic protein (Martin and Mulks 1999). Martin et al. reported that the protein reacted strongly with convalescent sera from swine infected with *A. pleuropneumoniae* and weakly with sera from swine vaccinated with a killed bacterin (Martin and Mulks 1999).

Spots 11 and 12 were match to *S. suis* CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain containing protein. The enzymes containing CHAP domain are involved in attacking the bacterial cell wall. CHAP domain is often found in association with other domains that cleave peptidoglycan (Stentz et al. 2009). Some members of this superfamily are important antigens in pathogenic bacteria and might represent drug and/or vaccine targets (Rigden et al. 2003). Some proteins containing CHAP domain were identified as immunogenic proteins in SS2 in our previous study (Zhang and Lu 2007b). Spot 15 was identified as peptidoglycan-binding LysM. In addition to binding specifically to peptidoglycan, proteins containing LysM domains have other enzyme activities (peptidase, chitinase, esterase, reductase or nucle-

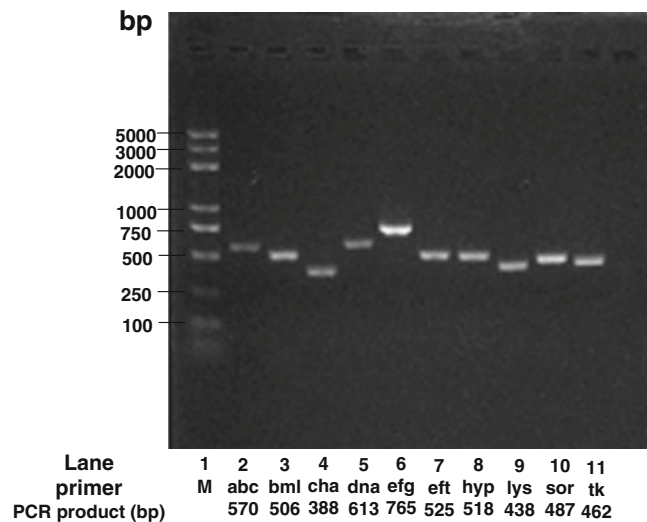


Fig. 2 PCR analysis of genes of immunogenic secreted proteins from SS9 strain GZ0565. Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 are primers abc, bml, cha, dna, efg, eft, hvp, lvs, sor, and tk, respectively, that were used for amplification; Lane 1, DNA markers

Table 4 Distribution of genes encoding immunogenic proteins in SS9 strains

SS9 strains	Detected gene of protein										
	ABC transporter substrate-binding protein - maltose/maltodextrin	ABC transporter periplasmic protein	CHAP domain containing protein	molecular chaperone DnaK	translation elongation factor G	Elongation factor Tu	hypothetical protein SSU98_2184	Peptidoglycan-binding LysM	sortase	Thymidine kinase	
GZ0565	+	+	+	+	+	+	+	+	+	+	
GD0606	+	+	+	+	+	+	+	+	+	+	
SH0626	+	+	+	+	+	+	+	+	+	+	
SH0627	+	+	+	+	+	+	+	+	+	+	
22083	+	+	+	+	+	+	+	+	+	+	
SH040917	+	-	+	+	+	+	-	-	-	+	
SH89	+	-	+	+	+	+	-	-	-	+	
JX041226	+	-	+	+	+	+	-	-	-	+	
NI0607	+	+	+	+	+	+	+	+	+	+	
SZ0608	-	+	+	+	+	+	+	+	+	+	
CZ0609	+	+	+	+	+	+	+	+	+	+	
YC0504	+	-	+	+	+	+	-	-	-	+	
XZ0503	+	+	+	+	+	+	+	+	+	+	
TZ0510	+	+	+	+	+	+	+	+	+	+	

+ : Corresponding sequence amplified; - : Could not amplify corresponding sequence

otidase) or act as antigens (Desvaux et al. 2006). The LysM domain is 40 residues long, and there are generally multiple tandem copies (between 1 and 6) localized in the N-terminal or C-terminal protein region (Desvaux et al. 2006). LysM-containing proteins may be secreted proteins, membrane proteins, lipoproteins or proteins bound to the cell wall (Buist et al. 2008). Several LysM-containing proteins are involved in bacterial pathogenesis (Andre et al. 2008). In our previous study, the LysM-containing protein was identified as putative virulence-associated factors whose gene was not found in the genome of SS9 strain SH040917 isolated from a healthy pig (Wu et al. 2008a). The *S. agalactiae* immunogenic protein (Sip), containing LysM domain, was able to protect mice against *S. agalactiae* infection throughout the experiment; whilst the incomplete Sip (NcoSip), lacking LysM domain, could only provide the lower protection (Vidova et al. 2009).

Translation elongation factors are responsible for two main processes during protein synthesis on the ribosome. Elongation factor-Tu (EF-Tu) mediates the entry of the aminoacyl tRNA into a free site of the ribosome. Elongation factor-G (EF-G) is responsible for the translocation of the peptidyl-tRNA from the A-site to the P-site (peptidyl-tRNA site) of the ribosome (<http://www.ebi.ac.uk/interpro/IEntry?ac=IPR004540>). Spot 4 was matched to *S. suis* EF-Tu. EF-Tu was identified as immunogenic protein in *Bacillus cereus* group (Delvecchio et al. 2006), and SS2 (Zhang and Lu 2007a; Zhang et al. 2008). Spot 2 was matched to *S. suis* EF-G. It was identified as immunogenic protein in *Bacillus cereus* group (Delvecchio et al. 2006).

Spot 13 matched the *S. suis* thymidine kinase. It is an important enzyme in the pyrimidine nucleotide salvage pathway and catalyzes the formation of thymidylate from thymidine using ATP as a phosphate donor (McNab 1996). Spot 1 matched the *S. suis* molecular chaperone DnaK. DnaK, homolog heat shock protein 70 (hsp70), plays an important role in the folding and unfolding or translocation of proteins, as well as in the assembly and disassembly of protein complexes (Zugel and Kaufmann 1999). It was identified as secreted protein by proteomic analysis in SS2 (Zhang and Lu 2007b). Hsp70 might be a good vaccine candidate, which was reported in *S. pneumoniae* (Hamel et al. 1997), *Mycobacterium tuberculosis* (Lowrie et al. 1997), and SS2 (Zhang and Lu 2007b). Spot 16 was matched to hypothetical protein SSU98_2184 of *S. suis*, which has no additional annotation. Although no function could be assigned, the presence of it could be interesting for investigating vaccine development of SS9. The immunogenic nature of these ten proteins is a novel observation in SS9.

PCR analysis was performed with ten primer pairs targeted to the genes of the identified proteins. The results confirmed that the primer pairs employed were able to

amplify genomic sequences from strain GZ0565 (Fig. 2). The distribution of these genes among SS9 strains is summarized in Table 4.

These immunogenic proteins, whose genes are reasonably conserved among SS9 strains, are expressed during a natural infection and may provide a function essential to pathogenesis. They are predicted to be a good target for vaccine development.

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