Comparative Proteomic Analysis of *Streptococcus suis* Biofilms and Planktonic Cells That Identified Biofilm Infection-Related Immunogenic Proteins

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Abstract

*Streptococcus suis* (SS) is a zoonotic pathogen that causes severe disease symptoms in pigs and humans. Biofilms of SS bind to extracellular matrix proteins in both endothelial and epithelial cells and cause persistent infections. In this study, the differences in the protein expression profiles of SS grown either as planktonic cells or biofilms were identified using comparative proteomic analysis. The results revealed the existence of 13 proteins of varying amounts, among which six were upregulated and seven were downregulated in the *Streptococcus* biofilm compared with the planktonic controls. The convalescent serum from mini-pig, challenged with SS, was applied in a Western blot assay to visualize all proteins from the biofilm that were grown in vitro and separated by two-dimensional gel electrophoresis. A total of 10 immunoreactive protein spots corresponding to nine unique proteins were identified by MALDI-TOF/TOF-MS. Of these nine proteins, five (Manganese-dependent superoxide dismutase, UDP-N-acetylgalactosamine 1-carboxyvinyltransferase, ornithine carbamoyl-transferase, phosphoglycerate kinase, Hypothetical protein SSU05_0403) had no previously reported immunogenic properties in SS to our knowledge. The remaining four immunogenic proteins (glyceraldehyde-3-phosphate dehydrogenase, hemolysin, pyruvate dehydrogenase and DnaK) were identified under both planktonic and biofilm growth conditions. In conclusion, the protein expression pattern of SS, grown as biofilm, was different from the SS grown as planktonic cells. These five immunogenic proteins that were specific to SS biofilm cells may potentially be targeted as vaccine candidates to protect against SS biofilm infections. The four proteins common to both biofilm and planktonic cells can be targeted as vaccine candidates to protect against both biofilm and acute infections.

Introduction

*Streptococcus suis* (SS) is a major worldwide pathogen and colonizes the respiratory tract of pigs, particularly the tonsils and nasal cavities [1]. SS is believed to be a normal inhabitant of several ruminants [2]. SS binds to the extracellular matrix (ECM) proteins, including fibrinogen and collagen [3], of endothelial and epithelial cells [4,5]. Some studies have demonstrated that SS has the ability to form biofilms [6,7]. The biofilm mode of growth affords SS several advantages over its planktonic counterparts, including the capability of ECM to trap nutrients and protect against both antimicrobial agents and the host immune responses [6,7]. Our previous studies indicate that SS maybe achieve persistent infections in vivo by forming biofilms [8] and hence SS infections might be difficult to treat. Biofilms play a key role in the pathogenesis and persistence of several bacterial infections [9]. It has been postulated that an altered metabolism and changes in gene expressions and protein amounts in biofilms may be responsible for drug resistance, cell adherence and virulence. Recent results indicate that biofilm cells have an active, although altered cell metabolism [10,11]. Considerable investigation is required to gain a better understanding of biofilm formation. Previous studies have investigated different immunogenic components of planktonically grown SS proteins; e.g., secreted or cell wall associated proteins using immunoproteomic assays [12,13,14,15]. Zhang *et al.* reported that 11 membrane-associated proteins and nine extracellular proteins are immunogenic proteins using the hyperimmune or convalescent serum of minipigs [12,13]. Geng *et al.* identified 32 proteins with high immunogenicity of which 22 were not previously reported [14]. Zhang *et al.* identified a total of 34 proteins by immunoproteomic analysis, of which 15 were recognized by both hyperimmune sera and convalescent sera [15]. At present, little is known about proteins targeted by the host immune system in the case of biofilm-mediated infections. Identifying those SS proteins that are targeted by the host immune system would increase the understanding of host defense mechanisms and help to identify novel means of diagnosis and treatment for pigs with persistent infections. Identification of these immunogenic antigens is necessary for effective vaccine design and
to understand the molecular mechanisms that control biofilm formation by SS. In this study, the differences in the whole cell protein expressions of SS cultivated under biofilm versus planktonic conditions were investigated. We utilized a convalescent mini-pigs model of challenged SS and an in vitro biofilm growth system to identify the immunogenic antigens of SS biofilm infections. We identified several proteins unique to SS grown as biofilms and planktonic cells by employing two-dimensional gel electrophoresis (2DGE) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF-MS) analysis.

**Materials and Methods**

**Bacteria and Culture Conditions**

SS2 strain clinical isolate HA9801 was used in this study. This strain was isolated by our laboratory in Jiangsu, China in 1998 and has the ability to form biofilms [8]. For biofilm cultures, SS was grown in THB medium (Oxoid, Wesel, Germany) supplemented with 1% fibrinogen in 100 mm polystyrene petri dishes at 37°C for 24 h. The supernatant was then removed and the plates were rinsed twice with 50 mM Tris/HCl (pH 7.5). Biofilms were detached by scraping. Cells were sonicated for 5 min (Bransonics 220; Branson Consolidated Ultrasonic Pvt Ltd, Australia), followed by centrifugation at 12,000 × g for 10 min at 4°C and the supernatant was discarded. Cell pellets were washed twice with 50 mM Tris-HCl (pH 7.5) by resuspending pellets with vortexing and collected by centrifugation 12,000 × g for 10 min at 4°C. SS planktonic cell was grown in 300 Erlenmeyer flasks containing 100 ml of the above culture medium at 37°C for 24 h. Planktonic cells were pelleted and washed as described above.

**Extraction of Proteins from SS Cells**

Protein was extracted from SS cells as described by Rathsam [16] with minor modifications. Briefly, the SS cell pellets from biofilm and planktonic cultures were resuspended in buffer (Tris-HCl, MgCl₂, 50% sucrose) supplemented with 1000 U/ml Mutanolysin (Sigma) and were incubated for 90 min at 37°C. The spheroplasts were collected and resuspended by sonication on ice at 100W for 90 cycles of (5 s on, 10 s off) using a sonication buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 65 mM DTT), and incubated at 25°C for 30 min. The cell debris and unbroken cells were removed by centrifugation at 10,000 × g for 30 min at 25°C. The proteins in the supernatant were precipitated using 10% TCA at 4°C for 30 min. Precipitated protein was collected by centrifugation at 10,000 × g for 10 min at 4°C and washed twice with chilled acetone. The final pellet was air-dried. The dried pellet was dissolved in sample preparation solution, then incubated for 30 min at 25°C (vortexed every 10 min) and centrifuged at 10,000 ×g for 20 min at 25°C. Before rehydration, the supernatant was treated with a 2-D Clean-up Kit (GE Healthcare) using a stepwise voltage gradient to 80 kVh. Before the second dimension, strips were equilibrated for 2×15 min in equilibration buffer (6 M urea, 2% SDS, 30% glycerol, 0.05 M Tris–HCl pH 8.3), containing 1% DTT and 4% iodoacetamide, respectively. SDS-PAGE was carried out vertically in an Ettan DALT II system (GE Healthcare) using 12.5% polyacrylamide gels. Resolved proteins were stained with Coomassie Brilliant Blue G-250 stain for identifying the protein bands. All experiments were performed in triplicate. Reproducibility of the 2DGE was verified by analyzing the same samples at least three times on independent gels. Three replicate gels from three independent experiments were analyzed for each growth condition. The gels were analyzed using the Image Master Platinum 5.0 software (GE Healthcare). The normalized protein amount for each protein spot was calculated as the ratio of that spot volume to the total spot’s volume on the gel. Either Student t-test (P < 0.05) or a threshold of 2-fold change was used to determine significant difference between the two groups.

**Preparation of Convalescent Sera**

Five specific pathogen free mini-pigs were injected with SS (1.0×10⁶ CFU/mL, 1 mL/pig, intramuscularly). As a control, preimmune sera were collected from mini-pigs before SS injection. Twenty days after the first injection, the survivor was again injected with second (identical) dose of SS. Serum was collected seven days after the second injection. The OD of the serum from pig injected with SS2 was 0.93±0.15 and the OD of the preimmune sera was 0.26±0.05. The titers of the convalescent sera were evaluated by ELISA (unpublished protocol), and the sera with high titer was selected for subsequent experiments. All animal experimental protocols were approved by Science and Technology Agency of Jiangsu Province (SYXK-SU-2010-0005).

**Western Blotting**

Protein samples from the 2DGE were transferred onto a PVDF membrane (GE Healthcare) using a semi-dry blotting apparatus (TE77, GE Healthcare) for 2 h at 0.63 mA/cm². After transfer, the membrane was blocked with 100 mM Tris, 150 mM NaCl, 0.05% Tween-20 (TBST), containing 5% dry milk powder for 2 h. The blocked-membrane was then incubated with sera from either preimmune or convalescent mini-pigs (1:1000 dilution) for 2 h at room temperature with gentle agitation. The membrane was washed three times with TBST buffer for 10 min per wash and incubated with horseradish peroxidase-labeled Staphylococcal protein A (Boster, Nanjing, China), (1:5000 dilution) in blocking buffer for 1 h with gentle agitation. The membrane was washed as described above. The membranes were incubated with DAB substrate (Tiangen, Nanjing, China) for 10 min. Each sample was analyzed three times by western blot.

**Mass Spectrometry Analysis of Protein Spots and Database Searches**

Differential expression spots and immune-reactive proteins were excised from the 2-D gels and sent to the Shanghai Applied Protein Technology Co. Ltd for trypsin in-gel digestion and MALDI-TOF-MS analysis. Protein spots with a low Mascot score were further analyzed using MALDI-TOF/TOF-MS to confirm identity. Data from MALDI-TOF-MS and MALDI-TOF/TOF-MS analysis were used in a combined search against the NCBI nr protein database using MASCOT (Matrix Science) with the parameter settings of trypsin digestion, one max missed cleavages, variable modification of oxidation (M), and peptide mass tolerance for monoisotopic data of 100 ppm. Originally, the MASCOT server was used against the NCBI nr for peptide mass fingerprinting (PMF). The criteria used to accept protein identifications were...
Reverse Transcription (RT)-PCR

Total RNA was isolated from SS grown as biofilms and planktonic cells for 24 h with an E.Z.N.A.™ bacterial RNA isolating kit (Omega, Beijing, China) following manufacturer’s directions. The RNA was subjected to DNase I (Promega, Madison, USA) treatment to remove DNA contamination. The cDNA synthesis was performed using the PrimeScript™ RT reagent kit (TaKaRa, Shanghai, China) following manufacturer’s instructions. Reactions were carried out in triplicate. An ABI 7300 RT-PCR system was used for relative qRT-PCR. Relative mRNA amounts and expression ratios of selected genes were normalized to the expression of 16S rRNA amounts and fold changes were calculated as described by Gavrilin et al. [17]. A specific primer set was used to analyze GAPDH (F; 5’-GTATGTCGGCTATGATC-3’ and R; 5’-TCATAGCAGCGTTTACTTCTTCAGC-3’), MRP (F; 5’-CAAGGAAAGTGAACAGAACGAGC-3’ and R; 5’-TAGTCGTGCCAAACCTGAGTAGCG-3’), and 16S rRNA (F, 5’-GTGCGGAACGGGTGAGTAA-3’ and R, 5’-CTTGGTAATCCCAGAATTGAACGG-3’). PCR products were analyzed by gel electrophoresis. The qRT-PCR results were confirmed by the SYBR Premix Ex Taq™ Kit (TaKaRa, Shanghai, China) following manufacturer’s instructions. Reactions were carried out in triplicate. An ABI 7300 RT-PCR system was used for relative qRT-PCR.

Results

Comparative Proteomics

2DGE of proteins from SS grown as biofilms or planktonic cells was performed to characterize the differences in protein expression between the two groups. The representative 2DGE images of biofilm and planktonic cells are provided in Figure 1. The majority of proteins were distributed in the range of pI 4–7 (Figures 1A and B). A total of 15 major protein spots were different between SS grown as biofilms or planktonic cells. MALDI-TOF-MS or MALDI-TOF/TOF-MS analysis identified 15 protein spots corresponding to 13 individual proteins. The probability score for the match, molecular weight (MW), isoelectric point (pI), number of peptide matches and the percentage of the total translated ORF sequence covered by the peptides were used as confidence factors in protein identification.

The proteins that were upregulated by more than two-fold included glyceraldehyde-3-phosphate dehydrogenase (GAPDH), UDP-N-acetylg glucosamine 1-carboxyvinyltransferase 2 (MurA), pyruvate dehydrogenase E1 component (PDH), ornithine carbamoyltransferase (OTC), hypothetical protein SU05_0403 and enolyl-CoA hydratase (Table 1). The proteins that were downregulated included ABC transporter periplasmic-binding protein (MntC), fructose-bisphosphate aldolase (FBA), dpyr, BAA, muramidase-released protein (MRP), triosephosphate isomerase and elongation factor Tu (ET-Tu) (Table 2).

Immunoreactive Proteins

Ten immunoreactive protein spots were observed on the immunoblot of SS biofilm whole-cell proteins (Figure 2B) that matched the protein spots observed in the 2DGE gel (Figure 2A). When the blot was probed with preimmune sera, no specific immunoreactive protein spots were observed (Figure 2C). A total of 10 immunoreactive protein spots, corresponding to nine unique proteins, namely GAPDH, MurA, PDH, OTC, manganese-dependent superoxide dismutase (SodA), hypothetical protein SU05_0403, molecular chaperone DnaK, hemolysin and phosphoglycerate kinase were identified (Table 3). Of these nine proteins, five (SodA, MurA, OTC, SSU05_0403, and phosphoglycerate kinase) have not been previously reported as immunoreactive proteins in SS to our knowledge. The remaining four immunogenic proteins (hemolysin, GAPDH, PDH and DnaK) have been identified in both planktonic and biofilm growth conditions in previous reports [12,13,14,15].

Confirmation of Comparative Proteomics Results by Quantitative Real-time PCR

Quantitative real-time PCR was performed on two selected genes to confirm the results of comparative proteomics analysis. We selected one upregulated gene (GAPDH) and one downregulated gene (MRP) in SS grown as biofilms. The qRT-PCR results confirmed the results of comparative proteomic analysis. SS grown as biofilms had 2.2 times higher GAPDH mRNA (P < 0.01) and
### Table 1. Proteins with increased expression levels in the SS biofilm, identified by MALDI-TOF/TOF MS.

| Spot no. | Protein identified | BLASTX similarity matched protein/species/identity score | Theoretical MW/PI | Experimental MW/PI | Mascot score | No. of Peptides matched | Coverage (%) | Fold change | Mean | SD | P value |
|----------|--------------------|-------------------------------------------------------|-------------------|-------------------|--------------|------------------------|--------------|-------------|------|----|---------|
| BF4      | gi|146317813| Glyceraldehyde-3-phosphate dehydrogenase | 35648/5.37 | 35000/5.40 | 280 | 24 | 62 | 2.5033 | 0.1955 | 0.006 |
| BF5      | gi|146317813| Glyceraldehyde-3-phosphate dehydrogenase | 35648/5.37 | 35000/5.45 | 279 | 24 | 67 | 2.2967 | 0.1595 | 0.005 |
| BF6      | gi|253752311| UDP-N-acetylgalactosamine 1-carboxyvinyltransferase 2 | 44720/5.28 | 41000/5.30 | 87 | 13 | 34 | 2.0667 | 0.0929 | 0.003 |
| BF7      | gi|253752506| Putative pyruvate dehydrogenase E1 component, alpha subunit | 35240/5.25 | 36000/5.15 | 166 | 15 | 51 | 2.0833 | 0.1518 | 0.006 |
| BF8      | gi|146318280| Ornithine carbamoyltransferase | 37832/5.26 | 40000/5.15 | 183 | 18 | 49 | 2.5067 | 0.1665 | 0.004 |
| BF10     | gi|146318058| Hypothetical protein SSU05_0403 | 31597/5.49 | 38000/5.00 | 304 | 21 | 86 | 2.2067 | 0.0611 | 0.001 |
| BF14     | gi|146319463| Enolase-CoA hydrolase | 28643/5.31 | 25000/5.30 | 179 | 16 | 55 | 2.6367 | 0.3415 | 0.014 |

a) gi number in NCBI.
b) Theoretical PI was calculated using AnTheProt (http://antheprot-pbil.ibcp.fr/).
c) Mascot score obtained for the peptide mass fingerprint (PMF). The significance threshold was 70.
d) Number of peptides that match the predicted protein sequence.
e) Percentage of predicted protein sequence covered by matched peptides.
f) Differential protein expression (fold change) of corresponding protein between *Streptococcus suis* planktonic and biofilm proteome.

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### Table 2. Proteins with decreased expression levels in the SS biofilm, identified by MALDI-TOF/TOF MS.

| Spot no. | Protein identified | BLASTX similarity matched protein/species/identity score | Theoretical MW/PI | Experimental MW/PI | Mascot score | No. of Peptides matched | Coverage (%) | Fold change | Mean | SD | P value |
|----------|--------------------|-------------------------------------------------------|-------------------|-------------------|--------------|------------------------|--------------|-------------|------|----|---------|
| FY1      | gi|146320941| ABC transporter periplasmic-binding protein | 36322/4.76 | 38500/4.60 | 208 | 18 | 58 | 3.8467 | 0.3384 | 0.005 |
| FY2      | gi|146320177| Fructose-bisphosphate aldolase | 31136/4.90 | 33000/4.85 | 201 | 16 | 49 | 2.6467 | 0.1823 | 0.004 |
| FY4      | gi|27651368| Dpr | 19583/4.91 | 22000/4.90 | 193 | 5 | 32 | 2.1667 | 0.2205 | 0.012 |
| FY5      | gi|1218040| BAA | 16523/6.98 | 145000/4.75 | 123 | 8 | 42 | 2.2366 | 0.1060 | 0.002 |
| FY6      | gi|189037416| Elongation factor Tu | 44727/4.87 | 39000/4.80 | 119 | 10 | 24 | 2.1733 | 0.2082 | 0.010 |
| FY7      | gi|146318185| Triosephosphate isomerase | 26907/4.68 | 26000/4.70 | 91 | 11 | 36 | 2.5733 | 0.2059 | 0.006 |
| FY8      | gi|225625045| Mucomidease-released protein | 135693/4.87 | 140000/4.80 | 158 | 9 | 52 | 2.4333 | 0.0950 | 0.001 |
| FY9      | gi|225625045| Mucomidease-released protein | 135693/4.87 | 140000/4.85 | 158 | 9 | 52 | 2.3800 | 0.1769 | 0.005 |
| FY10     | gi|146318184| Elongation factor Tu | 44727/4.87 | 34000/4.85 | 174 | 11 | 33 | 3.4200 | 0.2563 | 0.004 |

a) gi number in NCBI.
b) Theoretical PI was calculated using AnTheProt (http://antheprot-pbil.ibcp.fr/).
c) Mascot score obtained for the peptide mass fingerprint (PMF). The significance threshold was 70.
d) Number of peptides that match the predicted protein sequence.
e) Percentage of predicted protein sequence covered by matched peptides.
f) Differential protein expression (fold change) of corresponding protein between *Streptococcus suis* planktonic and biofilm proteome.

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0.3 times lower MRP mRNA amounts ($P < 0.05$) than SS grown as planktonic cells (Figure 3).

**Discussion**

In this study, the differences in the whole-cell protein expressions of SS grown under either biofilm or planktonic conditions were analyzed to reveal several differences in protein expressions between the two groups. Thirteen proteins, which showed differential expression under conditions of biofilm growth, were identified. Of the 13 proteins, six proteins were up-regulated and seven proteins were down-regulated in the biofilm proteome. Similar results have been demonstrated using other bacteria [18,19,20,21]. For example, nine proteins are up-regulated in the *Streptococcus mutans* biofilm cells compared with the planktonic cells [16]. Similarly, Alen *et al.* reported that eight proteins are up-regulated and four proteins are down-regulated in the *Nisseria meningitidis* biofilm [22]. In this study, though some other proteins were either down-regulated or up-regulated between the two groups, we only chose the 13 proteins because these 13 proteins were consistently different between triplicate gels. Using proteins from the biofilm cells and immunoblotting with convalescent sera, nine immunogenic proteins were identified. Only a limited number of proteins were identified, which may be due to serum being collected at early stages of infection in this study. Serum collected at late stages of infections identifies more protein spots [23].

Although bacteria in biofilms exhibit persistence in spite of sustained host defenses, little is known about the host immune response to biofilm infections. Protein expression in biofilms grown *in vivo* cannot be easily studied because it is difficult to extract bacterial proteins from *in vivo* grown biofilms. Certain antibodies may prevent biofilm development. For example, an antibody to an outer membrane protein in *Pseudomonas aeruginosa* was recently shown to inhibit biofilm formation by interfering with adhesion to the surface [23]. We employed a system in which mini-pigs were challenged with SS. By collecting sera from these mini-pigs during the course of infection and utilizing these sera to probe immunoblots of protein isolated from the *in vitro*-grown biofilm, we were able to visualize those immunogenic proteins that were present during biofilm infection. Though there are studies describing the immunogens present on the surface of planktonic SS, the data presented in this paper are the first to describe biofilm-specific proteins recognized by host antibodies. We found 10 immunoreactive spots that corresponded to nine individual immunogenic proteins. It was very interesting that five identified immunogenic proteins were up-regulated in the *Streptococcus* biofilm. A similar result has been found in *S. aureus*, where approximately 76% of the immunogenic proteins were upregulated in at least one of the stages of biofilm formation during *in vitro* growth [23]. Previous studies have evaluated the immunogenicity of SS proteins in planktonic growth conditions [15,14,15]. However, these studies failed to detect the biofilm-associated antigens found in this work, with the exception of hemolysin, GAPDH, PDH and DnaK. The above four common immunogenic proteins were identified in both growth conditions and hence could be promising vaccine candidates to prevent both biofilm infectious and acute infections. The remaining immunoreactive proteins in SS2 found in this study have not been previously reported to our knowledge.
Future studies should focus on identifying the role of GAPDH, MntC, OTC, FBA and PDH in biofilm formation, because Puttamreddy reported that biofilm formation and cellular adherence to epithelial cells are interlinked [24]. A previous study showed that these proteins could mediate cell adherence. GAPDH and MntC mutant strains confirmed the speculation [25]. Therefore, it is reasonable to think that other proteins might be involved in biofilm formation of SS. Study of the other proteins is ongoing in our laboratory to check if they are related to biofilm formation.

GAPDH is a glycolytic enzyme responsible for the conversion of glyceraldehyde 3-phosphate into 1,3-diphosphoglycerate. GAPDH is a SS surface protein and mediates cell adhesion and plays an important role in bacterial infection and invasion [26,27]. GAPDH was upregulated in SS grown as biofilms. Similarly, biofilms of Pseudomonas aeruginosa [28] and Staphylococcus xylosus [29] upregulate GAPDH. This also resembles the regulation of the enzyme in E. coli K12 under microaerobic conditions [30], which is indirectly linked to oxygen limitation in biofilms. Furthermore, SS mutants with GAPDH knocked-out had decreased ability to form biofilms (data not shown). It has also been reported that GAPDH is an immunogenic protein found on the cell wall of SS [15]. GAPDH is reported in the development of subunit vaccines against Edwardsiella tarda [31,32], Streptococcus pneumoniae [33] and Bacillus anthracis [34].

The protein from spot BF8 matched SS OTC. OTC is a key enzyme in the urea cycle and detoxifies ammonium produced from amino acid catabolism [35,36]. In Bacillus cereus, OTC was upregulated in biofilm cells at 18 h of culture. This may be indicative of oxygen depletion in microcolonies, or alternatively, it may indicate that the attached cells were preparing for growth within a biofilm before the conditions became anoxic. OTC is a putative adhesin for Staphylococcus epidermidis [37] and has been identified as an immunogenic protein from the outer surface protein preparations of S. agalactiae, S. pyogenes and Clostridium perfringens [38].

MntC is part of the MntABC transporter and is involved in oxidative stress defense in Nisseria gonorrhoeae and Nisseria meningitidis.
The N. gonorrhoeae MntC knock-out is more sensitive to oxygen killing, and accumulate less manganese than the wild type [39]. Furthermore, the gonococcal MntC knock-out have reduced intracellular survival and have reduced ability to form biofilms [25]. MntC facilitates biofilm formation of Gonococci, and affects the colonization of mice [40]. Allen et al. reported that biofilm formation is almost completely abrogated in the MntC mutants of Neisseria meningitides [22].

PDH converts pyruvate to acetyl coenzyme A, which is subsequently used in the tricarboxylic acid cycle to generate NADH, ATP, and reduced flavin adenine dinucleotide [41]. Welin et al. [42] and Korithoski et al. [43] used 2DGE to reveal that PDH is upregulated 2.5-fold in S. mutans biofilm cells. PDH is thought to play a role in the binding to fibronectin [44]. PDH is an important part of the cytoskeleton of M. pneumoniae and is linked to cell adhesion [45]. PDH is highly immunogenic in other bacterial species, such as N. meningitidis [46], Mycoplasma capricolum [47] and M. hyopneumoniae [48]. Recently, PDH has been tested as a DNA vaccine against M. mycoides subsp. mycoides, the causal agent of contagious bovine pleuropneumonia [49].

The upregulation of SodA involved in detoxification of ROS was in line with proteomic and microarray studies in biofilms of other bacteria; e.g., Staphylococcus aureus and Neisseria meningitidis [10,22]. SodA has a role in the protection of group A streptococcus challenge [50]. A similar result was shown with Listeria monocytogenes [51], Brucella abortus [52] and Escherichia coli [53]. Recombinant SodA elicits strong antibody responses in mice [53].

MurA is a key enzyme involved in bacterial cell wall peptidoglycan synthesis and a target for the antimicrobial agent, fosfomycin. Increased expression of MurA in the biofilms may contribute to the increased drug resistance [54].

The BLASTx search identified IP11 as molecular chaperone DnaK, IP12 as hemolysin and BF13 as phosphoglycerate kinase. DnaK is an important immunogen in S. pneumoniae [55] and S. pyogenes [56]. Hemolysin is a secreted protein and is a bacterial virulence factor [57]. Phosphoglycerate kinase is a major outer surface protein of S. suis. The above three proteins have been reported to be immunogenic in SS [13].

In this study, most of the downregulated genes such as FY1, FY2, FY4, FY5, FY6, FY7, and FY8 are likely to be involved in protein synthesis or encode membrane proteins/transporters (Table 2). This reduced level of expression may indicate a limited bacterial growth rate and that the SS organisms in biofilm environments have limited but more specific metabolic activity. Among the down-regulated genes was FY2 which represents fructose-bisphosphate aldolase. Fructoses are extracellular storage compounds and can act as binding sites for bacterial adhesion [58,59]. Extracellular fructans play a role in sucrose-dependent bacterial adherence and biofilm accumulation. To down-regulate this sucrose-dependent cell–cell adhesion, biofilm formation gene in biofilm cells makes bio-economic sense since sucrose is absent in the environment. FBA and MRP are virulence factors in a variety of organisms [60]. The expression of virulence factors in the planktonic cells will make the planktonic cells more virulent and, therefore, cause acute infections than biofilm cells [61]. In our previous study, biofilm cells had lower virulence when compared to planktonic cells in an animal model. In addition some virulence genes were downregulated in biofilm cells [8]. Changes in the structure of the bacteria may alter the expression levels of virulence genes. Biofilm cells are wrapped by a polysaccharide complex, which would influence the virulence factors secreted from the bacteria.

Author Contributions
Conceived and designed the experiments: YW LF WZ. Performed the experiments: YW GL. Analyzed the data: ZW LY. Contributed reagents/materials/analysis tools: WZ JS. Wrote the paper: WY LY.
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