Original Article

Differentiation of Secreted Proteins from a Virulent and an Avirulent Strain of *Streptococcus suis* Serotype 2

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Abstract

The present study aimed to identify specific and upregulated proteins in virulent SS2 strain that might confer pathogenicity. We performed a comparative proteomic assay to analyze the difference in secretome between virulent SH-08 strain and avirulent SH-YH07 strain. Virulence of tested strains was determined using porcine and zebrafish models. The supernatant from bacterial cultures was collected at early exponential phase (OD_{600} = 0.4). Proteomic assay identified 52 proteins from the virulent strain supernatant and 29 proteins from the avirulent strain supernatant. Twenty-three proteins were found in the culture medium from both strains. Among these common proteins, eleven were upregulated by the virulent strain compared to the avirulent strain as shown by a label-free quantitative strategy. The mRNAs of all differentially secreted proteins were analyzed by real time PCR to determine if the transcription of these proteins was also different. Subsequently, the mRNA levels of differentiated proteins were further analyzed in two other virulent strains (HA9801, ZY05719) and one avirulent strain (T15). This investigation demonstrated major differences in the secretome between a pathogenic and a nonpathogenic strain of SS2.

Keywords: extracellular protein, label-free proteomics, *Streptococcus suis* serotype 2, virulent factor

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บทคัดย่อ

ความแตกต่างของโปรตีนที่หลั่งจากเชื้อ *Streptococcus suis* serotype 2 ชนิดรุนแรงและไม่รุนแรง

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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาโปรตีนที่มีความจำเพาะและถูกเหนี่ยวนำให้เพิ่มขึ้นจากสายพันธุ์ก่อโรครุนแรง SS2 ที่อาจนำไปสู่การก่อโรค ใช้การทดสอบโปรตีนเปรียบเทียบ (a comparative proteomic assay) เพื่อวิเคราะห์ความแตกต่างของโปรตีนคัดหลั่ง (secretome) ระหว่างสายพันธุ์ก่อโรครุนแรง SH-08 และไม่รุนแรง SH-YH07 การกำหนดความรุนแรงของสายพันธุ์ที่ทดสอบ ใช้สุกรและปลาม้าลายเป็นต้นแบบศึกษา โดยเก็บส่วนใสที่ได้จากการเพาะเลี้ยงแบคทีเรียในช่วงต้น (early exponential phase; OD_{600} = 0.4) จากการวิเคราะห์ด้วย proteomic assay พบโปรตีน 52 ชนิดจากส่วนใสที่ได้จากสายพันธุ์รุนแรง และโปรตีน 29 ชนิดจากส่วนใสที่ได้จากสายพันธุ์ที่ไม่รุนแรง และสามารถพบโปรตีนที่เหมือนกันจำนวน 23 ชนิดจากทั้งสองสายพันธุ์ จากจำนวนโปรตีนเหล่านี้พบว่ามีโปรตีน 11 ชนิดที่ถูกเหนี่ยวนำให้เพิ่มขึ้นในสายพันธุ์รุนแรงเมื่อเทียบกับสายพันธุ์ที่ไม่รุนแรง โดยดูจาก label-free quantitative strategy จากนั้นทำการวิเคราะห์ mRNAs ของโปรตีนคัดหลั่งทั้งหมดโดยวิธี real time PCR เพื่อตรวจสอบความแตกต่างในระดับ transcription โดยmRNA ของโปรตีนที่แตกต่างนั้นถูกทำการวิเคราะห์ต่อไปจากสายพันธุ์รุนแรงอื่นๆ ได้แก่ HA9801, ZY05719 และจากสายพันธุ์ที่ไม่รุนแรง ได้แก่ T15 การศึกษาครั้งนี้แสดงให้เห็นถึงความแตกต่างที่สำคัญของโปรตีนคัดหลั่งที่ได้จากสายพันธุ์ก่อโรคและไม่ก่อโรคของ SS2

คำสำคัญ: โปรตีนภายนอกเซลล์ label-free proteomics *Streptococcus suis* serotype 2 ปัจจัยที่ทำให้เกิดความรุนแรง

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Introduction

Streptococcus suis (*S. suis*) is a zoonotic swine pathogen responsible for porcine meningitis, arthritis, pneumonia, endocarditis, encephalitis, polyserositis, and abscesses. *S. suis* serotype 2 (SS2) is the most common of the 32 identified serotypes. It has been documented in more than 20 countries, and has caused many human fatalities following streptococcal toxic shock syndrome (STSS) (Lun et al., 2007; Wertheim et al., 2009). The two large-scale outbreaks of SS2 human infection in China have posed significant public health concerns (Tang et al., 2006). Although there has been research on the pathogenesis of SS2, the pathogenesis is not yet fully understood. A few proteins, including muramidase-released protein (MRP), extracellular factor (EF), and suilysin (SLY), are possible markers for virulence. However, some nonpathogenic SS2 strains can also produce these proteins, while some SS2 strains isolated from diseased pigs were negative for these proteins (Gottschalk et al., 1998; Fittipaldi et al., 2009). Therefore, differences in virulence may not be determined solely by the differential expressions of MRP, EF, or SLY.

Extracellular proteins produced by bacterial pathogens play an essential role in host infection by facilitating host-pathogen interactions. The proteome of virulent SS2 has been well studied (Jing et al., 2008), and the differences in the secretomes between pathogenic and nonpathogenic SS2 and SS9 strains have also been investigated (Zhang and Lu, 2007; Wu et al., 2008[a]) by using 2 dimensional electrophoresis (2DE). However, this strategy suffers some drawbacks of identification of multiple proteins present in a single spot, identification of proteins at both extremes of the isoelectric point and molecular weight range (Gygi et al., 2000). Alternatively, LC-MS-based quantification methods combined with certain isotopic labeling have emerged to quantify peptides in mixtures of varying complexity. Although these approaches provide the greatest accuracy, isotopic labeling is relative expensive, laborious and potentially dangerous (Hughes et al., 2006). Label-free LC-MS quantification methods are based on the measurement and comparison of the MS signal intensities of peptide precursor ions. The improvement of ultra-performance liquid

chromatography (UPLC) has greatly increased the reproducibility of label-free LC-MS quantification. This strategy has successfully been applied to determine the relative abundance of proteins in different samples (Wiener et al., 2004; Silva et al., 2005; Hughes et al., 2006). The goal of this study was to investigate the proteins differentially secreted between the virulent and avirulent SS2 strains by using a label-free quantitative proteomic method and further confirming with Q-PCR and Western blots.

Materials and Methods

Strains and bacterial culture: The experiment design was specified in Fig 1. Strain SH-08 was isolated from a dead pig with septicemia. Strain SH-YH07 was taken from an unvaccinated healthy pig. Two other virulent strains (HA9801 and ZY05719) and a reference avirulent strain (T15) were selected (Wang et al., 2011). The SS2 strains were grown in Todd–Hewitt broth (THB, Oxoid) and plated onto Columbia agar blood base and incubated at 37^0C. The known virulent markers (mrp, ef, sly) were detected by PCR assay. The primers used for detection were described in previous study (Fittipaldi et al., 2009). Genomic DNA was isolated by using traditional phenol-chloroform method. PCR was performed with 36 cycles of 94^0C, 1 min; 56^0C, 1 min; 72^0C 1 min, by 10 min extension for the last cycle. After electrophoresis, PCR product was purified and dissolved into distilled water.

Overnight cultures were started from a single colony of SH-08 or SH-YH07 and grown in 25 ml THB. On the following day, cultures were centrifuged and resuspended in 25 ml THB medium. A 1000-fold dilution was then used to inoculate fresh THB medium. Samples from these cultures were collected hourly during a 19 hour incubation. Growth of bacterial cultures was determined by measuring the optical density at 600 nm (OD600). Colony forming units (CFU) were calculated by plating samples onto blood agar base (performed in triplicate).

Animal challenge: This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. The protocol for animal injections was approved by the Shanghai Animal Management Committee (Permit No: SYXK2007-0025).

Virulence of strains was determined in SPF pigs (Chinese Barns miniature pigs) and zebrafish. Four-week-old pigs in three dose groups (three pigs per group) were intramuscularly injected with a dosage with $2.8x10^6$ CFU to $2.8x10^8$ CFU. Clinical signs of infected pigs were recorded twice daily over seven days, including rectal temperatures, appetite, respiratory conditions, neurological signs, swollen joints and lameness. The LD_{50} of tested strains was determined in the zebrafish model as previously described (Wu et al., 2008[a]). Cultures were collected and washed twice with phosphate buffered saline (PBS, pH 7.4). Zebrafish were anesthetized with tricaine methanesulfonate in a water bath for 1 min at a final concentration of 95 mg/ml. Fish received a dose between $1.8x10^3$ and $1.8x10^5$ CFU by intraperitoneal injection. Each dose group had ten fish and each trial was repeated twice. The control group was injected with PBS. The fish in each group were in individually raised in tanks at 30^0C. The LD_{50} values were calculated using the method of Reed and Muench (1938).

Preparation of secreted proteins: Proteins were collected and separated as previously described (Zhang and Lu, 2007; Jing et al., 2008). In order to avoid contamination by cytoplasmic proteins released upon cell lysis, supernatants from bacteria in the early exponential phase (OD_{600} = 0.4) were collected, centrifuged at 8,000 g for 20 min at 4^0C, filtered through 0.22 µm membranes to remove residual bacteria, and stored at -80^0C. For equalization, the volume of the supernatant tested was adjusted based on the CFU/ml value of each strain. Trichloroacetic acid (10% w/v) was added to the supernatant, and this mixture was incubated at 4^0C for 30 min. After centrifugation at 10,000g for 10 min at 4^0C, the pellet was washed four times with acetone. The final pellet was air-dried, and then dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 50 mM DTT, and 4% CHAPS pH 8.5 buffer with protease inhibitors.

Protein digestion: The total supernatant protein extracted from each sample was chemically reduced by adding 10 mM DTT for 45 min at 55^0C. Proteins were then carboxyamidomethylated in 55 mM iodoacetamide for 30 min at room temperature in the dark. Reduced proteins were digested in trypsin buffer (containing 25 ng/ml trypsin, 10 mM NH_4HCO_3, pH 8.0) at 37^0C for 12 hour. After digestion, the peptide mixture was acidified by addition of 10 µl formic acid before mass spectroscopic analysis.

LC-MS/MS: The digested peptides were separated and identified using a Finnigan LTQ mass spectrometer (ThermoQuest, San Jose, CA, USA) coupled to a Surveyor HPLC system (Thermo). A Microcore RP column (C18 Hypersil, Thermo) was used to separate the protein digests. Solvent A was 0.1% (v/v) aqueous formic acid, and solvent B was 0.1% (v/v) formic acid in 100% (v/v) acetonitrile. The gradient was held at 2% solvent B for 15 min, and then increased linearly to 98% solvent B over 120 min. The peptides were eluted from the C18 microcapillary column at a flow rate of 150 ml/min, and then electrosprayed directly into a LTQ mass spectrometer (spray voltage of 3.2 kV, capillary temperature of 200^0C). The full scan ranged from m/z 400 to 2000. Proteins were identified from the MS/MS raw data using SEQUEST software (University of Washington, licensed to ThermoFinnigan). To avoid false annotations within a single genome or divergent peptide sequences between strains, the SWISS-PROT database containing all Streptococcus species was selected for match searching. Positive identifications were filtered with the Xcorr (1+ ≥ 1.9, 2+ ≥ 2.2, 3+ ≥ 3.75) and DelCn (≥ 0.1). Nonspecific matches were eliminated by searching a reversed protein sequence database generated from *Streptococcus*. The false

positive rate was below 3%.

Label-free detection and quantitation of peptides: Peptide detection, elution profile comparison, background subtraction, and peptide quantitation were all performed on the full scan precursor mass spectra in automatic mode using DeCyder MS. The presentation of LC-MS spectra as 2-D signal intensity maps was used for a visual raw data quality assessment. The PepDetect module of the software was used for automated peptide detection, charge state assignments based on resolved isotopic peaks and consistent spacing between consecutive charge states, and quantitation based on the MS signal intensities of individual LC-MS analyses. The final step (quantitative comparison) was performed by matching peptides across different signal intensity maps against the PepMatch module. The intensity distributions for all peptides detected in each sample were used for normalization, and peptides were identified by importing Turbo SEQUEST search results into the Pep-Match module. The mass tolerance in the software was set to 0.5 amu and the retention time tolerance was set to 2 min.

Quantitative reverse transcriptase PCR: Based on the results generated from the comparative proteomic assay, mRNA level of all proteins highly expressed in the hypervirulent strain SH-08, including one protein that was also upregulated in the avirulent strain, were selected for real time PCR detection. The primers used are listed in Supplementary File 4. Total RNA was extracted using Trizol reagent (TaKaRa, Dalian, China). To remove residual DNA, all extracted RNA samples were treated with DNAse, and residual DNA content was checked by an RT-PCR assay without reverse transcription. The cDNAs were synthesized from mRNA using a commercial kit (Reverse Transcriptase XL, TaKaRa, Dalian, China). Twenty microlitre reaction contained 10 µl of 2x RT buffer, 0.2 µl of enzyme, 2 µl of RNA, 100 pmol primers. Reverse transcription was carried out at 42^0C for 60 min. Amplification was done in reaction with SYBR green dye with the program of 95^0C for 3 min, 35 cycles: 95^0C for 15 sec, 60^0C for 30 sec, 72^0C for 30 sec was used. All reactions were performed in triplicate with negative controls.

To normalize the amount of tested cDNA in each run, the C_t value of the endogenous control gene 16S rRNA was subtracted from the C_t value of each gene (ΔC_t = C_t test gene - C_t 16S rRNA). To compare the expression of each gene in the virulent and the avirulent strain, the ΔC_t value of the gene in SH-08 was subtracted from the ΔC_t value of the gene in SH-YH07 ($\Delta\Delta C_t$ = ΔC_t SH-08 –ΔC_t SH-YH07). The relative changes were calculated using the formula $2^{-\Delta\Delta Ct}$ as described by Livak and Schmittgen (2001). Means±SD for each gene expression value was determined from triplicate reactions.

To further validate the data derived from proteomic analysis, transcription of candidates with the largest differences in protein level (phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, elongation factor Tu, cyclic nucleotide phosphodiesterase) was analyzed in two other virulent strains (ZY05719 and HA9801) and one avirulent strain (T15).

Western blotting: Two proteins (phosphopyruvate hydratase and pyruvate kinase) that showed discrepancy in the results between proteomic and PCR analysis were selected for Western blotting. Proteins were expressed using a prokaryotic expression system. In brief, genes were cloned into a PET-28a vector and transformed into *E. coli* strain BL 21. Transformed cells were incubated at 37^0C until the OD_{600} reached 0.6. Recombinant protein expression was induced by adding 1 mM IPTG and incubating the cultures for another 3 hour at 37^0C. Fusion proteins were purified by affinity chromatography (GE Healthcare) in accordance with the manufacturer's instructions.

Hyperimmune sera for each protein studied were obtained from specific pathogen-free rabbits immunized with purified proteins emulsions in Freund's complete adjuvant and boosted with Freund's incomplete adjuvant. Secreted and cytoplasmic proteins (equalization based on CFU vaule) from the virulent and the avirulent strain were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Amersham Pharmacia Biotech). Membranes were blocked with 5% skimmed milk in PBS (pH 7.4) containing 0.05% Tween-20 (PBST) for 2 hour at room temperature. Membranes were incubated for 1 hour at room temperature with the specific anti-sera (1: 3000) and washed with PBST. A horseradish peroxidase-conjugated anti-rabbit IgG (1: 15000, Amersham Pharmacia Biotech) was added and membranes were incubated for 1 h at room temperature. After washing with PBST, membranes were incubated with a SuperSignal West Pico chemiluminescent substrate (PIERCE) and then exposed to Kodak film for 1 min.

Bioinformatics: SignaIP 2.0.b2 trained on data from Gram-positive prokaryotes was used to analyze the secretion signals of all confirmed proteins. Proteins with a putative signal peptide were further analyzed using PSORT I (http://psort.nibb.ac.jp/) trained on Gram positive bacteria to predict subcellular location.

Results

Pathogenicity of SH-08 and SH-YH07 confirmed by animal challenge

The genotype of each strain was confirmed by PCR assay. Results demonstrated the genotype of SH08, SH-YH07, HA9801 and ZY05719 are *mrp*$^+$*ef*$^+$*sly*$^+$, while the genotype of T15 was *mrp*$^+$*ef* - *sly*$^+$.

All pigs infected with SH-08 showed elevated body temperatures (40.8 ± 0.6^0C). Pigs receiving a dose of $2.8x10^8$ CFU of SH-08 showed typical central nervous system signs (ataxia and opisthotonus) and were all dead in the first 48 hours. Two pigs ($2.8x10^7$ CFU) developed inappetite, meningitis and lameness and died with in seven days. Pigs infected with $2.8x10^8$ CFU of SH-YH07

Table 1 Secreted protein confirmed both in virulent and avirulent strains

Protein name **Cellular location**	**Confirmed peptide Sequence**	**Fold change relative to SH-YH07 (Protein level, 2Log)**	**Fold change relative to SH-YH07**
Phosphoglycerate kinase	K.AQGIEIGNSLVEEDKLDVAK.A		
bacterial cytoplasm	K.IGVIENLLEK.A	5.17	2.92
	K.LGQDVAFIAGATR.G	2.11	
	K.VLPGLAALTEK.	2.96	
	R.DTDGEAVSEGFLGLDIGPK.S		
	K.EANAFAGYTEVR.D		
	K.SIIGGGDSAAAAINLGR.A		
	K.SLAPVAADLAAK.L		
Phosphopyruvate hydratase (enolase)	K.GINSFYNLK.K K.GLNSFYNLK.K		
bacterial cytoplasm	K.VQLVGDDFFVTNTDYLAR.G		
	R.IEDQLGEVAVYK.G		
	R.SGETEDSTIADIAVATNAGQIK.T		
	R.TSAEQIDYLEELVNK.Y		
	R.GLVTAVGDEGGFAPK.F	1.98	-2.5
N-acetyl-beta-hexosaminidase	K.GQPQVIQLPAPINAR.Y		
bacterial membrane	K.IPTDLNSYTPESVATLK.E		
	R.EAEGLGWYNLDQGLR.G		
	K.ENVTVVDNFVEATPAYAR.T		
	R.NNQAFVDGYLAEVK.A		
	K.QDSEYLVDWVR.I		
metalloendopeptidase	K.AAGAYTAFLSDEIR.I		
bacterial cytoplasm	R.LGAEPAQALIAEYK.A		
	R.TGGFTDLADEIEK.L		
	K.LGAGESQIDNLSQLSEK.T		
GAPDH	K.AIGLVIPELNGK.L	4.1	15.9
bacterial cytoplasm	R.VPVPTGSVTELVATLDK.K	3.72	
	K.VIEVDGEQLVK.V	1.73	
	R.IQNVEGVEVTR.I		
DnaK	K.FDELTYDLVER.T		
bacterial cytoplasm	R.AKFDELTYDLVER.T		
	R.IVNEPTAAALAYGLDK.T		
	R.FQLTDIPAAPR.G		
ABC transporter periplasmic protein	K.AAAGDFPGGQVLQFSLK.D		
bacterial membrane	K.AAIVTDIGGVDDR.S		
	R.SFNQSAWEGLQEWGK.A		
	K.SSNFVLASTIK.G K.SSNFVLASTLK.Q#		
Elongation factor Tu	K.QLDEGIAGDNVGVLLR.G	6.57	3.18
bacterial cytoplasm	R.VNDEIEIVGLQEEK.S	3.34	
	R.RLPSSVNQPK.D		
ABC transporter (maltose binding protein)	K.EAGVNYGVATIPTLVNGK.N		
bacterial membrane	K.FVDFLTATDQQK.A		
	R.VGSLGSEGQLSELTLADDSK.A #		
	K.AAAIIEGPWK.A		
	K.TFAELETLAK.D		
alcohol dehydrogenase	K.LVFENLESSVK.N		
bacterial membrane	R.TNAILLPYVIR.Y		
	R.KDLEEAFAFGAEGLVVPIVEK.V*#		
deoxyribose-phosphate aldolase	K.TGNYDLVLEDIK.A		
bacterial cytoplasm	K.VCTVIGFPLGANTPAVK.A		
arginine deiminase	K.IEGSELVRGRGGPR.C	1.8	-1.69
bacterial cytoplasm	K.LAAESLVTPEIR.E	1.5	
	R.DEGVEVLYLEK.L		
	K.FTIHPEIEGDLR.V		
elongation factor Ts	K.NAQFVELVNTTAK.V		
bacterial cytoplasm	R.IGVISVVDGGDETLAK.Q		
ORF6	K.GGQSVSGSFGLPK.K	0.18	-10
bacterial outside	R.EVWWSVKPNTSK.S	0.38	
MRP	K.ITAPFTIDPTK.N	3.43	3.48
bacterial membrane	K.AGSVNVNFVDINGK.V	1.31	

Protein name Cellular location	Confirmed peptide Sequence	Fold change relative to SH-YH07 (Protein level, 2Log)	Fold change relative to SH-YH07
	K.AVLEQVTSESPLLAGLGQK.E K.VVEGETVVTYVYR.K K.YGDVIVEYYDTDGK.Q R.GTDTTGFIELLTTSPTTYK.V R.NATPATAVETTYIYK.E R.YSLDNDSSTTAVLAELVSR.T R.VFTGYDYVATTTK.A		
cyclo-nucleotide phosphodiesterase bacterial membrane	R.QAIINYIIAEK.V K.QQLAGTSEANLPILSAAAPFK.A* K.VINPTADNNWTFTDSIK.G R.GDASAYTDIPAGPIAIK.N	2.76	6.91
EPF bacterial membrane	R.VGEDNYPFGPEGK.L K.DIPSDFSIENFNLK.D K.TTSNSALVIDSSNYK.H K.EGIVGYIQPK.T	1.3	6.97
pyruvate kinase bacterial cytoplasm	K.IAALITEGANVFR.F K.IVATLGPAVEIR.G K.NAQTLLNEYGR.L K.IPFPALAER.D	2.65	-2.38
glucose-6-phosphate isomerase bacterial membrane	K.IAENQAYQYAAVR.N K.PGFEELGAELNAR.L R.NIFETVVR.V	1.3	2.9
hypothetical protein SSU05_0940 bacterial cytoplasm	R.DQLQTAMYSTFIKR.Q	2.16	2.73
pneumococcal histidine triad A protein bacterial membrane	K.TAIYGFLKELYGEFAPQPK.V	0.87	-12.5
hypothetical protein SpyoM01000052 bacterial cytoplasm	K.TLSQAGELEKRLHQFR.Y	0.78	-7.69
glucosyltransferase bacterial membrane	K.NRSANPDSDPVPNYVFIR.A		

showed mild fever, inappetence and lassitude in the first three days. No death was observed with SH-YH07 infection.

Four days after injection with SH-08 strain, zebrafish mortality was 95% (19/20) after $1.8x10^5$ CFU, 40% (8/20) after $1.8x10^4$ CFU, and 20% (4/20) after $1.8x10^3$ CFU. Half of these deaths occurred within the first 12 hour. However, avirulent SH-YH07 infection induced no deaths. Accordingly, the LD_{50} value for SH-08 was calculated as $1.3x10^4$ CFU.

Protein identification and data processing

To avoid extensive cell lysis, bacteria were collected in the exponential phase of growth (OD_{600} = 0.4). The respective CFU values of SH-08 and SH-YH07 strains were $2.6x10^7$ and $1.7x10^7$ when collected, so 50 ml of SH-08 and 76.5 ml of SH-YH07 supernatant were used for the proteomic assay. Protein concentration of SH-08 and SH-YH07, confirmed by using bicinchoninic acid assay (BCA), was 183 µg/ml and 116 µg/ml, respectively.

Table 2 Proteins specifically confirmed in virulent strain

Protein name Cellular localization	Sequence	Peptide counts	Coverage Percentage
translation elongation factor G bacterial cytoplasm	K.GFEFENAIVGGVVPR.E K.VEANVGAPQVSYR.E R.HASDEEPFAALAFK.I R.LQANAHPIQLPIGSEDEFR.G R.VYSGVLNSGSYVLNTSK.G R.VYSGVLNSGSYVLNTSK.G	6	11.26%
hemolysin bacterial outside	K.GVDISGNAEYQDILK.N K.IAVPLDINFDAVNSGEK.Q K.LFAEGTTVEDLK.R R.ADQNLLDNNPTLISIAR.G K.AANIYPGALLR.A	5	13.20%
30S ribosomal protein S1 bacterial cytoplasm	K.VGETLELLVLR.Q K.VLAIDEVEGR.V K.DTDTVTYLVSK.K K.GGLAVEFEGLR.G	4	6.02%

Protein name **Cellular localization**	**Sequence**	**Peptide counts**	**Coverage Percentage**
6-phosphogluconate dehydrogenase bacterial outside	K.ELANSGINFIGTGVSGGEK.G K.ENNWNLPFGEIAK.I R.AENLPANLIQAQR.D	3	9.47%
ABC-type xylose transport system bacterial cytoplasm	K.ENSDGSVYALEFTGK.A R.GGAQGTAIYNNTIFSDSK.L R.SVSLDDIIIR.Q	3	1.39%
L-lactate dehydrogenase bacterial cytoplasm	K.DIIDDAFANPEIAAGVK.N R.TQGDAEDLSHALAFTFPK.K K.LYDWLQDNR.D	3	10.70%
transcriptional regulator/sugar kinase bacterial membrane	K.EGDDLALIVYR.N R.GNDAGVLGAASLVLK.- K.LAILTTEGEIQEK.W	3	8.15%
putative 5'-nucleotidase bacterial membrane	K.LLGEASLISAADTK.N K.NVEGVTFTDPVTEVNK.V K.TADLSAYEVVNPYSR.I	3	5.29%
glucan phosphorylase bacterial membrane	K.DSDTIIDLYDK.A K.ELIGDEYLTDATK.L K.ENLTLFLYPDDSDK.N	3	2.15%
response regulator bacterial cytoplasm	K.TSSADEILAAIR.K K.VALGEYAIETEVEK.K R.VMLVDDHEM*VRLGLK.S	3	7.36%
oligoendopeptidase F bacterial membrane	K.NSGGYCTYIPDFK.S K.TFSSDVIPLLQK.E K.SGGSCTPLETALLIGADISTDK.P	3	4.64%
ABC transporter ATPase bacterial cytoplasm	K.IELEEIIPSSR.K R.EIGNDLLTVENLK.V	2	2.16%
ribonucleases G and E bacterial membrane	K.LIAPTTDSVITDNEVSK.E R.EANGEELPVLDTAQNTK.E	2	5.66%
sigma 54 modulation protein bacterial cytoplasm	K.VATGQVFTDELVEQTGEEVK.V R.REDGDLGLLEVR.Q	2	17.78%
anchored protein FneC bacterial membrane	K.GEKGDPGQQGIPGPK.G K.GDTGKDGKDGQPGPQGPK.G	2	5.25%
oxygen-independent coproporphyrinogen III bacterial cytoplasm	R.NRGPIQHYLKSIR.E R.SHNQAQIYETIATLK.E	2	7.47%
ribosome-associated protein Y bacterial cytoplasm	K.VATGQVFTDELVEQTGEEVK.V R.REDGDLGLLEVR.Q	2	17.78%
fructose-bisphosphate aldolase bacterial membrane	K.EVVELAHAK.G K.VNVNTECQIAFANATR.A	2	8.53%
50S ribosomal protein L1 bacterial cytoplasm	K.AAGADFVGEDDLVAK.I K.LYSVEEAVALAK.E	2	14.52%
triosephosphate isomerase bacterial cytoplasm	K.NPQEAQAFVEAIAGK.L R.DVVAADFGQEVADK.V	2	11.60%
arginyl-tRNA synthetase bacterial membrane	K.AAVEADPISELLK.L K.LADTESWEIIK.H	2	4.27%
hypothetical protein SSU05_0020 bacterial membrane	K.SQLSWVGPYWGDAK.Q R.YDASSYPVGECTWGVK.S	2	7.18%
glutamyl-tRNA synthetase bacterial cytoplasm	R.DTNTLQFIEDYR.K R.EQFIQLFDENR.L	2	4.96%
30S ribosomal protein S2 bacterial cytoplasm	K.LADQAYEFIR.D R.DAAANDAVILFVGTK.K	2	9.69%
manganese-dependent superoxide dismutase bacterial cytoplasm	K.AFFEVINWDK.V	1	5.29%
LysM repeat protein bacterial membrane	K.SGDTLSEIAETYNTTVEK.L	1	9.38%
Asparagine--tRNA ligase bacterial membrane	R.VFDFGPVFR.A	1	2.55%
competence protein CelA bacterial membrane	K.PAPQTSVKETNLQAEVAAVSKDSSTGK.E	1	12.50%
Phosphoglycerate mutase 1 bacterial cytoplasm	R.YAHLDDSVIPDAENLK.V	1	8.47%

To positively identify each protein, at least two different peptides were confirmed in a single assay, or one tryptic peptide was identified in all three replicates. We identified and confirmed 52 proteins from SH-08 and 29 proteins from SH-YH07. Among these, 23 proteins were detected in both strains (Table 1). The remaining 29 proteins were defined as specific to the virulent strain (Table 2). In contrast, only six proteins were found to be specific to the avirulent strain (Table 3). By using SignaIP 2.0 and PSORT I, 25 proteins from SH-08 (48%) were predicted to be secreted or membrane-associated. Likewise, 15 proteins from SH-YH07 (51.7%) were likely secreted or membrane-associated.

Identification of upregulated and downregulated proteins

Expression analysis by DeCyder MS software yielded the relative differences in expression for all identified proteins from the two investigated strains. A threshold 1.3-fold increase and 0.5-fold decrease was used as the cut-off for significant upregulation and downregulation (Wiener et al., 2004; Silva et al., 2005; Hughes et al., 2006). All protein hits with a confidence of > 95% and identified in all three runs were regarded as differentially expressed. Eleven proteins were confirmed to be upregulated in SH-08 strain relative to SH-YH07, while only one upregulated protein was confirmed in SH-YH07 culture supernatant (Table 1).

Verification of differential proteins by QRT-PCR and Western blotting

The gene transcription of 11 upregulated and the one downregulated protein expressed in SH-08 were analyzed by real time PCR. Results between proteomic and real time PCR assay were compared (Fig 1). Discrepant results were apparent for three proteins. Phosphopyruvate hydratase was overexpressed by SH-08 relative to SH-YH07 (1.98-fold increase in protein) but there was a 2.5-fold decrease in mRNA. Similarly, arginine deiminase was overexpressed by 1.5 fold, but there was a 2-fold decrease in mRNA, while pyruvate kinase was overexpressed by 2.65 fold, but the mRNA was 2.5-fold lower in the virulent SH-08 strain relative to avirulent SH-YH07. Detailed results (primers, Ct value, amplification curve) derived from real time PCR are listed in Supplementary File 1. This contradiction was further validated by immunoblotting in the case of enolase and pyruvate kinase. Results also showed similar protein concentrations in the cytoplasmic fraction but reduced protein in the supernatant from the avirulent strain, suggesting that virulent SS2 bacteria have an enhanced capacity for releasing some intracellular proteins through a non-lytic process (Fig 1). For further validation of the data derived from the proteomic assay, mRNA levels of phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, nucleotide phosphodiesterase, and elongation factor Tu (with at least 2.5 fold increase in virulent strain protein) were also analyzed in two other virulent strains (HA9801, ZY05719) and one avirulent strain (T15). Results showed significantly elevated transcription of all tested genes in these two virulent

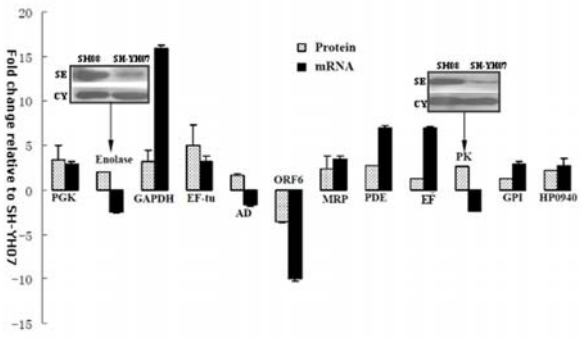

Figure 1 Differentially expressed proteins as determined by proteomics, quantitative PCR, and Western blotting. Fold changes in all upregulated and downregulated proteins in the virulent strain relative to avirulent strain are illustrated in the column diagram. Standard deviations calculated from three replicates are provided at the top of each bar. Immunoblots of enolase and pyruvate kinase are reproduced. The labels SE and CY indicate the secreted and cytoplasmic fraction of tested strains, respectively.

Table 3 Proteins specifically confirmed in avirulent strain

Protein and cellular localization	Sequence	Peptide Counts	Coverage
pullulanase **bacterial membrane**	**K.LGFLLINQNNPDLAGNK.T** **R.GDTSTLAGNDSQGWLISK.R** **R.VLGDFNQENAGYTLK.Y** **K.SLAEWNSDLADTDPSYR.I** **K.VVATNLLSETVVDSETR.I** **K.NDVVIAYQTIASNGDR.Y** **K.YGAYVDIPLSNGLDSK.L** **K.LGFLLINQNNPDLAGNK.T**	**7**	**2.54%**
hyaluronidase **bacterial membrane**	**K.TIFNDIDFSK.S** **R.NNWEDISLANK.R**	**2**	**4.02%**
putative acetate kinase **bacterial outside**	**K.NVFGNYGDISTAESK.V** **R.VLVIPTDEELVIAR.D**	**2**	**19.08%**
membrane-fusion protein **bacterial membrane**	**K.NILIPVSAVVPEEDK.N** **R.YDATELQSALDTAVR.G**	**2**	**7.79%**
SNF2 family protein **bacterial cytoplasm**	**R.DYDRFVRQFGYLNVSVNR.N**	**1**	**0.87%**
DNA gyrase A **bacterial cytoplasm**	**R.ASETDAEAQAELM*SKFKLSER.Q**	**1**	**2.71%**

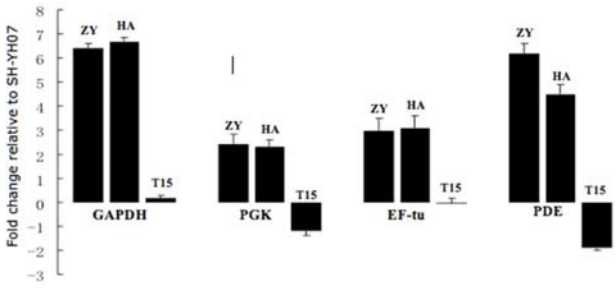

Figure 2 Transcription of four selected proteins in virulent strains of ZY05719 and HA9801 and in the avirulent strain T15. Fold changes on mRNA level of tested proteins in two other virulent and a reference avirulent strains relative to SH-YH07 were determined by quantitative PCR. Standard deviations calculated from three replicates are provided at the top of each bar. Labels ZY and HA indicate the virulent strains of ZY05719 and HA9801.

strains compared to SH-YH07. In contrast, similar mRNA levels of elongation factor Tu and glyceraldehyde-3-phosphate dehydrogenase and lower mRNA levels of phosphoglycerate kinase and nucleotide phosphodiesterase were found in the avirulent strain T15 (Fig 2).

Discussion

Although cultures were collected in the early exponential phase to avoid interference from cytoplasmic proteins releasing during cell lysis, half of the identified proteins in the present study were predicted to be cytoplasmic. However, some cytoplasmic proteins have been confirmed to be actively secreted through an as yet undetermined transport mechanism, including glyceraldehyde-3-phosphate dehydrogenase and enolase (Wu et al., 2008[a]). Secretomes of virulent and avirulent strain of *S. suis* serotype 2 and 9 have been investigated by using a gel-based strategy. In these studies, only seven and thirteen proteins specific for virulent SS2 strain and SS9 strain were identified (including MRP, EF, enolase, nucleotidase), respectively. In the present study, a label-free quantitative proteomic method was performed. There were 29 proteins identified as specific in virulent SS2 strain. Moreover, eleven extracelullar proteins with significant upregulation in virulent strain were also confirmed. The potential association of the new identified proteins with SS2 pathogenesis was discussed. Although the association has not been experimentally confirmed, results derived from this study are expected to provide an inventory for further studies of virulent factor identification and SS2 pathogenesis.

Orthologous proteins

There were 23 extracellular proteins expressed by both the virulent SH-08 and avirulent SH-YH07 strains. Of these, 11 proteins were highly expressed in SH-08 relative to the avirulent strain while only one unidentified protein was relatively overexpressed in SH-YH07. Some well-recognized virulent factors with obvious signal peptides such as MRP and EF were detected in the supernatants from both strains. Determination of the genotypes for MRP and EF has been performed in some epidemiological studies (Gottschalk et al., 1998; Fittipaldi et al., 2009). However, variation in the expression of these proteins was reported in those studies, which suggested that phenotyping rather than genotyping should be performed to determine the virulence of the strain. In the present study, quantitative proteomic analysis and real time PCR confirmed that the expression of MRP was upregulated in the virulent strain by 3.4 fold and 3.5 fold, respectively. Proteomic study confirmed that secreted EF protein was mildly elevated in the virulent stain (1.3-fold), but that the EF mRNA level was significantly increased in the virulent strain.

The remaining nine upregulated proteins in SH-08 were predicted to be cytoplasmic, including phosphoglycerate kinase (PGK), phosphopyruvate hydratase (enolase), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor Tu (EF-tu), arginine deiminase, cyclic nucleotide phosphodiesterase (PDE), pyruvate kinase, glucose-6-phosphate isomerase (GPI), and one hypothetical protein (HP0940). GAPDH is the key enzyme in the glycolytic pathway, but has also been confirmed as a secreted or membrane-associated protein in S. suis (Zhang and Lu, 2007; Jing et al., 2008; Wu et al., 2008[a]). *S. suis* serotype 2 mutants deficient in GAPDH expression showed attenuated adhesion to host cells (Brassard et al., 2001). Thus, GAPDH may contribute to tissue invasion and destruction (Jobin et al., 2004). Notably, mismatch between protein and mRNA levels was comfirmed with enolase, arginine deiminase and pyruvate kinase, which indicated that the transportation of these cytoplasmic enzymes to the extracellular space through an undetermined (non-lytic) mechanism is more effective in the virulent strains. Enolase is a glycolytic enzyme with both cytoplasmic and membrane location (Pancholi and Fischetti, 1998). A potential contribution of enolase to the pathogenicity of *S. suis* was suggested by binding of plasmin(ogen), which allowed bacteria to acquire the proteolytic activity essential for tissue invasion (Esgleas et al., 2008; Baums and Valentin-Weigand, 2009[a]). Arginine deiminase, belonging to the Arginine–Deiminase system (ADS), is also a putative virulent factor for SS2. Recent reports demonstrated that expression of ADS enhanced survival of *S. suis* under oxygen and nutrient starvation, and in acidic environments (Benga et al., 2004; Gruening et al., 2006).

Unique proteins

There were 29 extracellular proteins confirmed to be specific to the virulent strain, but only six specific to the avirulent strain (table 2). PCR confirmed that genes of these unique proteins existed both in virulent and avirulent strains (data not shown). Therefore, the difference in transcriptional or translational regulation in virulent and avirulent strains might be responsible for the occurence of unique proteins. Some of the unique proteins of SH-08 have been confirmed to be virulence-associated or antigenic in previous studies, including SLY, 30S

ribosomal protein S1, L-lactate dehydrogenase, putative 5'-nucleotidase, and ribonucleases G and E (Zhang and Lu, 2007; Jing et al., 2008).

Suilysin is a well recognized virulent factor of *S. suis* and is cytotoxic to epithelial cells, brain microvessel endothelial cells, and macrophages (Norton et al., 1999; Charland et al., 2000; Segura and Gottschalk, 2002). Here, only SH-08 strain released SLY into the culture supernatant, though both tested strains were genotypically positive for SLY.

A previous study confirmed that the extracellular 5'-nucleotidase was unique to the culture supernatant of virulent *S. suis* serotype 9, suggesting that it is a putative virulence factor (Wu et al., 2008[b]). The contribution of 5′-nucleotidase to virulence may, like 2′3′ cyclic phosphodiesterases, relate to nucleotide salvage (TrÜlzsch et al., 2001). Ribonucleases G and E (RiGE) are membrane-associated proteins that are accepted antigens of SS2 (Zhang and Lu, 2007; Zhang et al., 2008). However, the function of RiGE related with virulence has not been recorded.

In response to environmental stress, some pathogens will produce many more ribosomal and protein-synthesis related proteins (Kiliç et al., 2010). In the present study, 30S ribosomal protein S1 and S2, 50S ribosomal protein L1, and EF-G were found only in the culture supernatant of the virulent strain, suggesting that the general elevation of protein biosynthesis in the virulent strain contributes to bacterial survival and to infection *in vivo*.

In contrast to SH-08, only seven proteins were confirmed to be specific in the culture supernatant of the avirulent strain SH-YH07. One putative virulence factor (hyaluronidase) was identified. Hyaluronidase is important for the pathogenesis of *S. suis* serotype 7 infection, but not crucial for *S. suis* meningitis and other invasive diseases, as highly virulent SS2 strains lack this activity (Baums and Valentin-Weigand, 2009[a]).

Conclusion

In summary, the present study investigated the difference in secretome between the virulent and avirulent SS2 strain. The proteins identified belonged to three categories: well recognized virulent factors (MRP, EF and SLY), proteins that enhance adhesion capacity (GAPHD, phosphoglycerate kinase, and phosphopyruvate hydratase), and ribosomal and protein-synthesis related proteins (nucleotide phosphodiesterase, 30S ribosomal protein S1 and S2, 50S ribosomal protein L1, EF-Tu, and 5'-nucleotidase). Our knowledge on upregulated and specific proteins in the virulent SH-08 strain might aid in understanding the pathogenesis of SS2.

Acknowledgments

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