Biochemical and Biophysical Research Communications 446 (2014) 997–1001

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Comparative proteome analysis of the extracellular proteins of two *Haemophilus parasuis* strains Nagasaki and SW114

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ARTICLE INFO

Article history:
Received 1 March 2014
Available online 19 March 2014

Keywords:
Comparative proteome
Haemophilus parasuis
Extracellular proteins
Quantitative PCR

ABSTRACT

This study used a comparative proteomics approach to distinguish between the two-dimensional electrophoresis profiles of extracellular proteins in Nagasaki and SW114. Protein spots were identified using matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry. The ten proteins unique to Nagasaki were putative adhesin AidA protein, putative extracellular serine protease (autotransporter) (771aa), putative extracellular serine protease (autotransporter) (780aa), protective surface antigen D15, 30S ribosomal protein S2, periplasmic serine protease do/hhoA-like protein, acid phosphatase, membrane protein, protein-disulfide isomerase, and iron ABC transporter substrate-binding protein. Meanwhile, the two proteins unique to SW114 were C4-dicarboxylate ABC transporter substrate-binding protein and peptide ABC transporter substrate-binding protein. Quantitative PCR was used to analyze the mRNA transcript levels of three randomly selected proteins. The *afuA*, *AidA*, and *ompD15* genes encoding iron ABC transporter substrate-binding protein, putative adhesin AidA protein and protective surface antigen D15 respectively demonstrated significantly higher mRNA transcript levels (39.606, 3.924, and 36.668, respectively) in Nagasaki than in SW114. These observations suggest the levels of differentially expressed proteins were directly proportional to their cellular mRNA levels. Three virulence-related proteins, namely, putative adhesin AidA protein, putative extracellular serine protease (autotransporter) (771aa) and putative extracellular serine protease (autotransporter) (780aa) were identified in Nagasaki.

1. Introduction

Haemophilus parasuis, a pleomorphic, gram-negative, nicotinamide adenine dinucleotide (NAD)-dependent rod in the family Pasteurellaceae, is a commensal organism of the upper respiratory tract of swines [1]. This bacterium is the causative agent of Glässers disease, which is characterized by fibrinous polyserositis, arthritis, and meningitis, or acute pneumonia and acute septicemia [2]. With the recent changes in production methods, diseases caused by *H. parasuis* have received increasing attention worldwide because of the serious economic losses that these diseases cause in the pig industry. To date, 15 serotypes with apparent differences in virulence have been recognized [3]. However, several nontypeable isolates exhibit high heterogeneity at the molecular level [4]. Among the 15 known serotypes, serotypes 4 and 5 are the most prevalent in many countries [5], and serotype 5 is more virulent than serotype 4. To date, a number of possible virulence-related factors investigated in the family Pasteurellaceae have been identified in *H. parasuis*, including lipopolysaccharide, capsular polysaccharide, fimbriae, outer membrane proteins, and iron acquisition systems [6]. However, little is known about the bacterial components that are involved in *H. parasuis* infection.

Bacteria secrete a large number of proteins to enable them to adapt and survive in their environment. Extracellular proteins are significant in the course of infection because of their remote action and direct connection with host cells. Extracellular protein analysis allows the identification of new putative virulence factors, which are pivotal for pathogen survival during host infection. Proteome analysis, a technique that combines two-dimensional (2D) electrophoresis and mass spectrometry (MS), is an extremely useful method to determine the pathogenicity factors in microorganisms [7]. The extracellular proteins in numerous pathogenic bacteria, such as *Pseudomonas aeruginosa* [8], *Bacillus anthracis* [9], and *Vibrio anguillarum* [10], have been analyzed via proteome analysis. In this study, a comparative proteomics approach was used to distinguish between the 2D profiles of extracellular proteins in two *H. parasuis* strains, namely, SW114 (non-virulent reference

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http://dx.doi.org/10.1016/j.bbrc.2014.03.049
0006-291X/

strain, serovar 3 [3]) and Nagasaki (virulent reference strain, serovar 5 [3]), in order to identify differently expressed extracellular proteins in the two strains.

2. Materials and methods

2.1. Bacterial strains and culture conditions

SW114 strain (non-virulent reference strain, serovar 3) and Nagasaki strain (virulent reference strain, serovar 5) were purchased from Bacteriology Research Laboratory, Animal Research Institute, Yeerongpilly, Australia. The two strains were grown in tryptic soy broth (TSB) (Difco; USA) or tryptic soy agar (TSA) (Difco; USA) at 37 °C and were both supplemented with NAD (10 mg/mL).

2.2. Growth curve of H. parasuis strains

A single colony was used for subcultivation in TSB overnight at 37 °C. On the following day, a 100-fold dilution of the initial culture was grown in fresh TSB. Bacterial cultures were sampled at 3-h intervals from the time of culture inoculation ("0 time") to a 33 h incubation period. Bacterial growth was monitored by measuring the optical density at 600 nm (OD600). Colony forming units (CFUs) were determined by plating appropriate serial dilutions onto TSA.

2.3. Preparation of extracellular proteins

Protein preparation was performed with a few modifications, as described by Zhou et al. [11]. In brief, the two strains were grown to mid-logarithmic phase (OD600 of 0.6) in TSB at 37 °C. To prevent proteolytic degradation of extracellular proteins, the protease inhibitor (Protease Inhibitor Cocktail, Roche) was added before the extracellular proteins were extracted. The bacterial cultures were centrifuged at 10,000*g* for 15 min at 4 °C. The supernate was collected, centrifuged at 4 °C for 20 min at 12,000*g*, and filtered through a 0.22 mm membrane to remove residual bacteria. Extracellular proteins were precipitated using trichloroacetic acid and acetone. The filtrate was mixed with prechilled 100% trichloroacetic acid to a final concentration of 10%, incubated in ice water for 2 h, and centrifuged at 10,000*g* at 4 °C for 10 min. The pellet was resuspended in 10 mL of prechilled acetone, washed three times, and air dried.

2.4. 2D gel electrophoresis

Extracellular proteins were dissolved in an immobilized pH gradient (IPG) rehydration/sample buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3–10, 65 mM DTT, 0.2% Bio-Lyte, 0.001% bromophenol blue) (Bio-Rad; USA) and centrifuged at 12,000*g* for 15 min at room temperature to remove undissolved materials. The protein content was determined using the Bradford protein quantitative kit (TIANGEN; China). A 17 cm Immobiline DryStrip (IPG, pH range 3–10; Bio-Rad) was rehydrated at 50 V for 12 h in an IPG rehydration/sample buffer containing 2 mg of the protein sample in a total volume of 350 μL. Isoelectric focusing (IEF) was performed using a Bio-Rad PRO-TEAN IEF cell (Bio-Rad; USA). Focusing was conducted by stepwise increases in the voltage, as follows: 250 V for 0.5 h, 1000 V for 1 h, 8000 V for 5 h, and 8000 V until 60,000 Vh. The temperature was maintained at 20 °C. After IEF separation, each IPG strip was washed in 5 mL of equilibration buffer I [0.375 M Tris–HCl (pH 8.8), 6 M urea, 2% SDS, 20% (v/v) glycerol, 20 g/L DTT] for 15 min and 5 mL of equilibration buffer II [0.375 M Tris–HCl (pH 8.8), 6 M urea, 2% SDS, 20% (v/v) glycerol, 25 g/L iodoacetamide] for an additional 15 min. The IPG strips were then placed over a 12% resolving polyacrylamide gel, and electrophoresis was performed in two steps at 10 °C: 120 V for 30 min and then at 240 V until the tracking dye reached the bottom of the gels. All gels were stained with Coomassie Brilliant blue G250 (Bio-Rad; USA). Gel evaluation and data analysis were performed using the PDQuest 8.0 program (Bio-Rad; USA). Three replicates were run for the samples.

2.5. MS analysis of protein spots and database searches

Spots unique to the Nagasaki and SW114 strains were excised from the 2D gels for tryptic in-gel digestion and MALDI-TOF/TOF-MS. Fasta was used for MASCOT (Matrix Science) with the parameter sets of trypsin digestion, one maximum missed cleavage, variable modification of oxidation (M), and peptide mass tolerance for monoisotopic data of 100 ppm. The criteria for accepting protein identifications were based on the protein score confidence interval (CI). Protein identification was assigned when the protein score CI was >95%.

2.6. Quantitative PCR detection

Total RNA was extracted from the exponentially growing *H. parasuis* (OD600 = 0.6) using the RNAprep Pure Cell/Bacteria Kit (TIANGEN, China) according to manufacturer instructions. The total RNA concentration was determined by measuring the absorbance at 260 nm (A260) and the RNA quality was assessed by determining the ratio of A260–A280 using agarose gel electrophoresis. Samples with RNA purity above 1.8 were used. Purified RNA samples were aliquoted and stored at −80 °C.

Total RNA was reversely transcribed into cDNA using a ReverAid First Strand cDNA synthesis kit (Fermentas, USA). The absence of chromosomal DNA contamination was confirmed by real-time PCR on the corresponding DNase I-treated RNA. Quantitative real-time PCR (qRT-PCR) was performed on a MiniOption™ Detector real-time PCR instrument using SYBR green I detection. The 16srRNA gene of *H. parasuis* was used as the internal control. Each reaction mixture contained 1 μL of 10-fold-diluted cDNA, 10 μL of RealMasterMix (SYBR Green) (TIANGEN), 0.5 μL of 100 nM of each sense and antisense primer, and 8 μL of H_2O. The gene *16srRNA*, *afuA*, *AidA* and *ompD15* forward and reverse primer sequences used are listed in Table 1.

The amplification profile consisted of 2 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 15 s at 54 °C, and 15 s at 68 °C. Fluorescence was monitored during each extension phase. At the end of the amplification cycle, a melting analysis was conducted to verify the specificity of the reaction. This analysis was performed by heating the amplification products from 55 °C to 95 °C at 0.5 °C/10 s and monitoring the decrease in fluorescence. The data represent the fold changes in mRNA expression relative to the control (SW114 strain), which was arbitrarily defined as 1. All experiments were performed in triplicate. For data analysis, the fold change in expression was calculated for each target gene using the CT method [12].

3. Results and discussion

3.1. General observations

A comparison of the extracellular proteins of the Nagasaki and SW114 strains was performed to identify differently expressed proteins. The supernatant proteins of the two strains were obtained at the mid-logarithmic phase of the growing culture (see Fig. 1). The Nagasaki strain gel contained 32 protein spots, whereas the number of protein spots in the SW114 strain gel was 25 (Fig. 2).

Table 1
Genes and primers used for qRT-PCR.

Gene	Protein	Forward/reverse primer sequence (5′-3′)
16srRNA	–	Forward: CGGGAAACTGTCGCTAAT Reverse: TGTGGCTGGTCATCCTCT
AfuA	Iron ABC transporter substrate-binding protein	Forward: GACAGTTGGCTTTGCTCAC Reverse: TCGCATTATCAAGGTTTCTC
AidA	Putative adhesin AidA protein	Forward: CAAGTGAACAAGCGACAAC Reverse: GATACAACGGTGGCATAAC
ompD15	Protective surface antigen D15	Forward: CGTCAGCAAGAAGGAGCAT Reverse: CGCCGTATTTGGGACATT

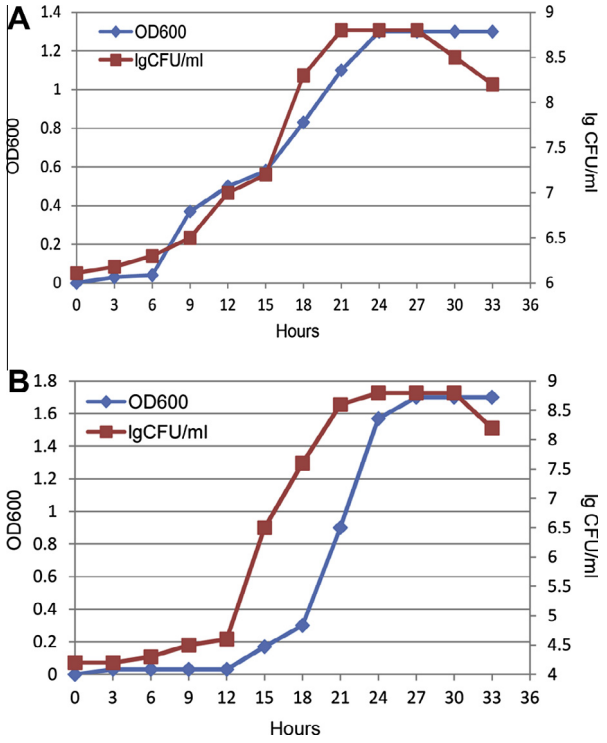

Fig. 1. Growth curves of *Haemophilus parasuis* strains. (A) Nagasaki and (B) SW114.

These results are consistent in at least three independent gel analyses per biological sample.

3.2. 2-D gel comparison

Protein spots observed at similar locations in both gels were marked with the same number and labeled in red. Protein spots that were unique to the SW114 strain were labeled in magenta, whereas spots unique to the Nagasaki strain were labeled in blue. All differential proteins were then characterized by MALDI-TOF/TOF-MS. The probability scores for the match, as well as the molecular weight, isoelectric point (pI), and protein score CI% were used as confidence factors in protein identification. Spots 0804, 0805, 0901, 4802, 5905, 6102, 6609, 7302, 7305, 7701, 7705, 8305, 9002, and 9205 were successfully identified. Spots 7305, 7701, and 7705 were unique to the SW114 strain, whereas the other 10 proteins spots, namely. 0804, 0805, 0901, 5905, 6102, 6609, 7302, 8305, 9002, and 9205, were unique to the Nagasaki strain. These 13 successfully identified spots corresponded to 12 individual proteins. The results are summarized in Table 2.

30S RPS2 (spot 6102) is involved in the formation of the translation initiation complex and possibly interacts with mRNA and several components of the ribosome. *Escherichia coli* RPS2 is essential for the binding of RPS1 to the 30S ribosomal subunit [13]. Ribosomal protein was also found in secreted proteins of Streptococcus suis according to Chengping Lu [14]. The periplasmic serine protease do/hhoA-like protein (spot 6609), which is involved in heat-shock response, chaperone function, and apoptosis [15], was identified from the culture supernate of several pathogenic bacteria, such as *P. aeruginosa* [16] and *Staphylococcus aureus* [17]. The peptide ABC transporter substrate-binding protein (spots 7701 and 7705) is an ABC-type dipeptide transport system involved in amino acid transport and metabolism [18]. Chengping Lu [14] also found proteins related to amino acid transport and metabolism when investigating the secreted proteins of Streptococcus suis. Spots 7701 and 7705 had the same molecular mass but slightly different isoelectric points, which may be attributed to phosphorylation. Iron ABC transporter substrate-binding protein (spot 9205) participates in inorganic ion transport and metabolism [19]. Further studies must be conducted to determine the function of this protein in *H. parasuis*. Meanwhile, protein-disulfide isomerases (spot 9002) function as protein disulfide isomerases and chaperones in the bacterial periplasm to promote the correct folding of secreted or surface-presented factors, such as toxins, adherence factors, and components of type III secretory systems [20]. These isomerases are considered as critical virulence factors.

Acid phosphatase (spot 7302) is a haloacid dehalogenase-like hydrolase that exhibits acid phosphatase activity, metal-ion binding, and transferase activity. A number of secreted phosphatases have evolved specialized functions that are relevant to microbial virulence [21]. C4-dicarboxylate ABC transporter substrate-binding protein (spot 7305) is an extracytoplasmic solute receptor for the tripartite ATP-independent periplasmic transporter [22]; however, the general function of this protein remains limited.

Adhesin AidA protein (spot 0804) is a member of an autotransporter family that consists of proteins secreted by the type V secretion system. Adhesin AidA protein was originally identified in diarrheagenic *E. coli* that showed diffuse adherence to epithelial cell surfaces and has been suggested as one of the afimbrial adhesins that mediate adhesion [23]. Another two potential autotransporters related to the type V protein secretion pathway were two putative extracellular serine proteases. Spot 0805 matched putative extracellular serine protease (autotransporter) (780aa). Meanwhile, spot 0901 matched the putative extracellular serine protease (autotransporter) (771aa). The amino acid sequences of the two extracellular serine proteases showed high similarities (73%), which suggest that these proteases were encoded by duplicate copies of the same gene, as reported by Xu et al. [24]. Autotransporters were a large and diverse superfamily of proteins produced by pathogenic gram-negative bacteria that were composed of an N-terminal passenger domain and a C-terminal beta domain. The beta domain anchored the protein to the outer membrane and facilitated transport of the passenger domain into the extracellular space [25,26]. This type V family of secreted proteins participate in a diverse range of host–pathogen interactions associated with virulence, such as adhesion, invasion, autoagglutination,

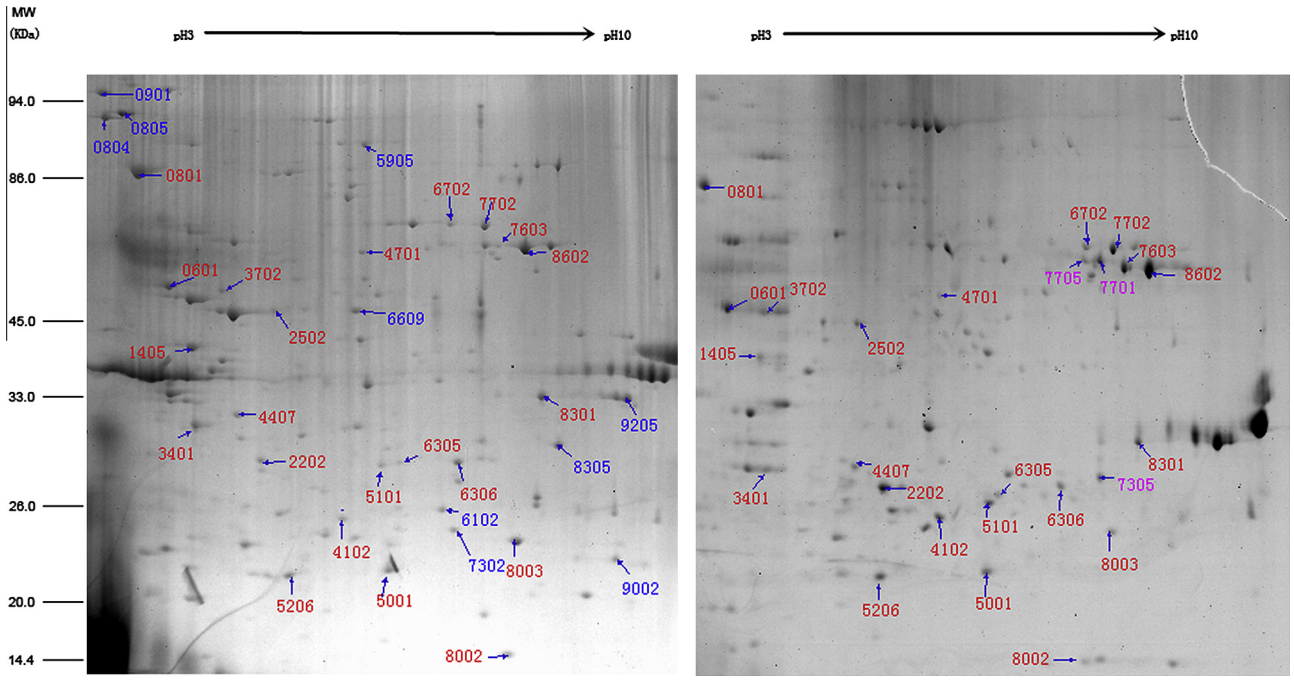

Fig. 2. Two-dimensional map of the extracellular proteins of (left) Nagasaki and (right) SW114.

Table 2
Protein spots identified by MALDI-TOF/TOF-MS.

NO.	Protein name	NCBI entry number	Mr, Da	pI	Protein score	Protein score C. I.%
Spots identified in Nagasaki						
0804	Putative adhesin AidA protein [*Haemophilus parasuis*]	gi|498484921	34754.9	5.81	80	99.937
0805	Putative extracellular serine protease (autotransporter), (780aa) partial [*Haemophilus parasuis*]	gi|498485396	51888.5	5.21	338	100
0901	Putative extracellular serine protease (autotransporter), (771aa) partial [*Haemophilus parasuis*]	gi|498485399	52235.7	4.73	278	100
5905	Protective surface antigen D15 [*Haemophilus parasuis*]	gi|498484330	90343.9	7.59	553	100
6102	30S ribosomal protein S2 [*Haemophilus parasuis*]	gi|501705533	26314.6	6.32	330	100
6609	Periplasmic serine protease do/hhoA-like protein[*Haemophilus parasuis*]	gi|498484672	48033.4	7.72	259	100
7302	Acid phosphatase [*Haemophilus parasuis*]	gi|491995100	26852.4	6.96	239	100
8305	Membrane protein [*Haemophilus parasuis*]	gi|491993949	29745.8	7.7	303	100
9002	Protein-disulfide isomerase [*Haemophilus parasuis*]	gi|491998344	25345.4	8.67	235	100
9205	Iron ABC transporter substrate-binding protein [*Haemophilus parasuis*]	gi|498484424	37754.7	8.8	339	100
Spots identified in SW114						
7305	C4-dicarboxylate ABC transporter substrate-binding protein [*Haemophilus parasuis*]	gi|498485445	34816.3	7.08	269	100
7701	Peptide ABC transporter substrate-binding protein[*Haemophilus parasuis*]	gi|491995571	57908	8.23	543	100
7705	Peptide ABC transporter substrate-binding protein[*Haemophilus parasuis*]	gi|491995670	59431.2	7.62	331	100

Table 3
Results of relative expression of gene *AfuA*, *ompD15* and *Aida* in Nagasaki than SW114.

Gene	Type	Expression	Std. Error	95% C.I.	P(H1)
16srRNA	REF	1.000			
AfuA	TRG	39.606	30.936–51.433	26.427–57.440	0.067
ompD15	TRG	36.668	26.246–51.593	21.331–63.610	0.060
Aida	TRG	3.924	3.521–4.618	3.105–4.833	0.034

P(H1) – Probability of alternate hypothesis that difference between sample and control groups is due only to chance.
TRG – Target.
REF – Reference.

inhibition of complement and degradation of IgA [27,28]. In 2009, Zhou et al. [29] investigated *H. parasuis* SH0165 through 2D electrophoresis and immunoblotting and found that the protective surface antigen D15 (spot 5905) is an effective immunogenic outer membrane protein. The membrane protein (spot 8305) is mainly involved in cell envelope biogenesis. Even though we tried to avoid cross-contaminations, outer membrane proteins were found in the extracellular proteome fraction, which was found when the proteomes of diverse microorganisms were investigated [30]. The outer membrane proteins were found in extracellular proteome fraction might be the result of cell lysis.

Of all the identified proteins, putative adhesin AidA protein, putative extracellular serine protease (autotransporter) (771aa) and putative extracellular serine protease (autotransporter) (780aa) have been proposed as putative virulence-associated factors of *H. parasuis*, and all the three proteins existed in the extracellular proteins of the virulent Nagasaki strain.

3.3. Transcriptional profiles of genes encoding differentially expressed proteins

The *afuA*, *AidA*, and *ompD15* genes demonstrated significantly higher mRNA transcript levels (39.606, 3.924, and 36.668, respec-

tively) in the Nagasaki strain than in the SW114 strain (Table 3). The mRNA expression of the three genes in the SW114 strain was arbitrarily defined as 1. The results showed that the three genes namely, *afuA*, *AidA*, and *ompD15,* as well as the their mRNA and their encoded proteins, exhibited a positive correlation. These observations suggest that the levels of differentially expressed proteins were directly proportional to their cellular mRNA levels.

Acknowledgments

This work was supported by the haemophflussuis and porcine contagious pleuropneumonia prevention technology research and demonstration project (201303034), which is financed by the public service sectors (Agriculture) research project special funds.

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