Comparative Proteome Analysis of Outer Membrane Proteins of *Bordetella bronchiseptica*

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ABSTRACT

Bordetella bronchiseptica (Bb) is an important pathogen in rabbits and is associated with a respiratory infectious disease, which is long lasting, repeatable and difficult to cure. Serious economic losses in rabbit husbandry are caused by the disease. The outer membrane (OM) is the barrier between a bacteria and the environment. Bb OMPs possibly contain virulence factors associated with disease. Proteomics that combines 2-DE with mass spectrometry has become a powerful tool to study the virulence factors of pathogenic microorganisms. In this research, the outer membrane proteins (OMPs) proteomics profiles were compared in two Bordetella bronchiseptica (Bb) strains, HB and RB. Bb strains were isolated from an infectious rhinitis rabbit and a healthy rabbit, respectively. The 50% lethal dose value (LD50) in mice of strain HB was 2.42×106 cfu/ml, while mice infected with RB had low mortalities. The OMPs were extracted by sodium carbonate treatment, and analyzed with two-dimensional gel electrophoresis (2-DE). The 2-DE gels of OMPs showed mostly protein spots ranging from 40 kDa to 70 kDa. 140 protein spots in RB and 187 in HB gels were detected. Among these proteins, 14 spots of RB showed lower intensity than the corresponding spot of HB, while 5 spots showed higher intensity. Moreover, 5 protein spots were observed only in HB. Different Protein spots were cut out and identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). Combined peptide mass fingerprinting PMF and MS/MS queries were carried out by the MASCOT search engine 2.2. The theoretical PI/Mw of proteins were computed by the PI/Mw tool (http://web.expasy.org/compute pi/), the functional categories of the identified proteins of Bb were sorted using COGnitor (http://www.ncbi.nlm.nih.gov/COG/old/ xognitor.html) and the subcellular location was predicted by PSORTb v.3.0 psort (http://www.psort.org/psortb/). These identified proteins belong to different classes: cell envelope biogenesis, outer membrane; cell motility and secretion; inorganic ion transport and metabolism; energy production and conversion; amino acid transport and metabolism; translation, ribosomal structure and biogenesis; posttranslational modification, protein turnover, chaperones; function unknown proteins and NO related COG. In conclusion, in our research, OMPs of two different virulence strains of Bb were analyzed via 2-DE and identified by MALDI-TOF-MS, in order to screen virulence proteins of Bb and find novel proteins associated with virulence. These identified proteins may be associated with virulence of Bb, however these findings still need further studies.

Key Words: *Bordetella bronchiseptica*, Outer Membrane Proteins, Virulence Factor, Proteomics

INTRODUCTION

Bordetella bronchiseptica (Bb) is an important pathogen in rabbits and is associated with a respiratory infectious disease, which is long lasting, occurs repeatedly and is difficult to cure. Serious economic losses are caused in rabbit husbandry by the disease. Moreover, Bb also can cause canine infectious tracheobronchitis and swine infectious atrophic rhinitis. Bb virulence were sorted into two types, the first includes adhesins, including filamentous hemagglutinin (FHA), pertactin (PRN), tracheal colonization factor (TCF) and fimbriae (FIM); the second includes toxins, including dermonecrotic toxin (DNT), tracheal cytotocin, adenylate cyclase toxin (AC-Hly), type-III secretion system and lipopolysaccharide (LPS). Bb colonizes in the cilia of the respiratory mucosa epithelial cells, and then proliferates and secretes virulence factors, such as DNT and AC-Hly, causing inflammation of the mucosal epithelial cells, hyperplasia, cilia loss and other degenerative diseases. The expression of these virulence factors is closely related to the environment of the pathogen and is regulated by two-component regulatory systems (TCSs). Bacteria can sense and respond to environmental stimuli

by TCSs, and the expression of related genes is controlled by TCSs (Laub et al. 2007).

The outer membrane (OM) is the barrier between a bacteria and the environment. One of its functions is to protect bacteria against undesirable environment. A lipid bilayer covers the cell and 50% of its mass is composed of proteins (Koebnik et al. 2000). The OMPs of bacteria are the interface of the interaction between bacteria and extracellular environment, playing an important role in drug resistance, pathogenicity and immunogenicity (Cordwell et al. 2001). Many OMPs in *E. coli* have been identified, including bacteriophage receptors, resistance to antibiotics, structural functions as well as adhesion, and invasion proteins (Poetsch et al. 2008). Therefore, Bb OMPs possibly contain virulence factors associated with disease.

Proteomics that combine 2-DE with mass spectrometry has become a powerful tool to study the virulence factors of pathogenic microorganisms (Jungblut et al. 2000). Thus in our research, OMPs of two different virulence strains of Bb (HB and RB) were analyzed via 2-DE and identified by MALDI-TOF-MS, in order to screen virulence proteins of Bb and find novel antigenic proteins associated with virulence.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Bb strain HB isolated from an infectious rhinitis rabbit and RB isolated from a healthy rabbit were used in this research. Bacteria of Bb were cultured on sheep blood agar (Hangzhou Tianhe Microganism Reagent Co, Ltd.) and also in tryptone soya broth (TSB, Oxoid) liquid medium containing 5% bovine calf serum in a rotary incubator shaker at a speed of 200 rpm at 37°C for the extraction of OMPs.

Extraction of OMPs sample

OMPs were extracted by the method of Molloy et al. with some modifications (Liao et al. 2009). Firstly, in the late phase of Bb (OD600~1.5), cells were centrifuged at 7000 × g (HITACHI, High-speed Refrigerated Centrifuge) for 10 min at 4°C. Secondly, the harvested pellets were washed 4 times with low salt PBS(3 mM KCI, 68 mM NaCI, 1.5 mM KH2PO4, 9 mM NaH2PO4) and the pellets were suspended in Tris-Cl [50 mM, pH 7.5, containing 1% protease inhibitor (GE Healthcare)]. Thirdly, the mixture prepared in the second step were ruptured at 2 W; pulse on, 2 s; pulse off, 2 s (MISONIX, sonicator® 3000) by sonication, till the OD600 decreased to 1/10 of the original. Cells and cellular debris unbroken were removed by centrifugation, at 9000×g for 10 min at 4°C. The supernatant was diluted 10-fold with ice cold Na_2CO_3 (0.1 M, pH 11), and stirred slowly in ice bath for 1 h. OMPs were collected by ultracentrifugation at 4°C in a ultracentrifuge (HITACHI) running at 100000×g for 1 h. The supernatant was removed, and the pellets were resuspended and washed in Tris-Cl (50 mM, pH 7.5) twice. The pellets were gained by centrifugation at 100000×g for 1 h at 4°C. Finally, the pellets were solubilized in a lysis buffer [5 M Urea, 2 M Thiourea, 2% (W/ V) CHAPS, 2% (W/ V) SB3~ 10]. The concentration of proteins was detected using GE Healthcare 2-D Quant Kit. The protein samples were packaged and stored at -70°C.

2-DE and Image Analysis

The OMPs samples (750 μg/strip) were mixed with lysis buffer (5 M Urea, 2 M Thiourea, 2% CHAPS, 2% SB3-10), containing 1% DTT, 0.5% IPG buffer and 0.002% bromophenol blue, and loaded on 13 cm Immobiline DryStrip™ IPG strips (linear 4-7 pH gradient, GE Healthcare, USA). The strips with OMPs samples were focused for 410 kVHrs on the Ettan IPGphor III system. Then the IPG strips were equilibrated for 15 min with 1% DTT and 2.5% IAA in equilibration buffer (6 M urea, 75 mM pH 8.8 Tris-HCl, 29.3% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue). After equilibration, the 2-DE was carried out on a 12.5% SDS polyacrylamide gel with the SE600 Ruby system (GE Healthcare, USA). Gels were stained with Coomassie Brilliant Blue G250. Gels were evaluated and analyzed by software of Imagemaster 2D Platinum 7.0 (GE Healthcare, USA). Spots of each gel of RB and HB were matched and the percent volume of

spots was compared. The protein spot levels were considered to have higher or lower volume when there was at least a 1.5-fold difference (Paes et al. 2008). At a minimum, triplicate gels of each sample were analyzed to check reproducibility.

Protein In-gel digestion

Protein spots were picked out from gels, destained in 200~ 400 µl 100 mmol/l NH_4HCO_3/30% CAN for 20 min, and washed in distilled water. Then these spots were lyophilized after kept in 0.2 M NH_4HCO_3 for 20 min. Each spot was digested overnight at 37°C, the ratio of the mass of trypsin and proteins analyzed was 1 : 20~1 : 100. The solution was transferred to a new tube, 60% CAN/0.1% TFA added, ruptured by sonication, lyophilized and desalted by Ziptip.

Analysis of MALDI-TOF MS and database searches

Protein identification was performed on a MALDI-TOF-TOF instrument (4800 proteomics analyzer, Applied Biosystems), whose parameters were set using the 4000 Series Explorer software (Applied Biosystems). Combined peptide mass fingerprinting PMF and MS/MS queries were carried out by the MASCOT search engine 2.2 (Matrix Science, Ltd.), with MS/MS fragment tolerance setting to 0.4 Da. GPS Explorer protein confidence index ≥ 95% were used for further manual validation.

Bioinformatics tools

The theoretical PI/Mw of proteins were computed by the PI/Mw tool (http://web.expasy.org/compute pi/), the functional categories of the identified proteins of Bb were sorted using COGnitor (http://www.ncbi.nlm.nih.gov/COG/old/xognitor.html) and the subcellular location was predicted via PSORT b v.3.0 psort (http://www.psort.org/psortb/).

RESULTS

Comparison of OMPs Profiles by 2-DE

The gels were submitted to image analysis using Imagemaster 2D Platinum 7.0. The 2-DE gels of OMPs showed mostly protein spots ranging from 40 kDa to 70 kDa. 140 protein spots in RB and 187 in RB gels were detected. Using analysis parameters as ratio >1.5, 14 spots of RB showed lower intensity than the corresponding spot of HB, while 5 spots showed a somewhat higher intensity. Moreover, 5 protein spots were observed only in HB (Figure 1 B).

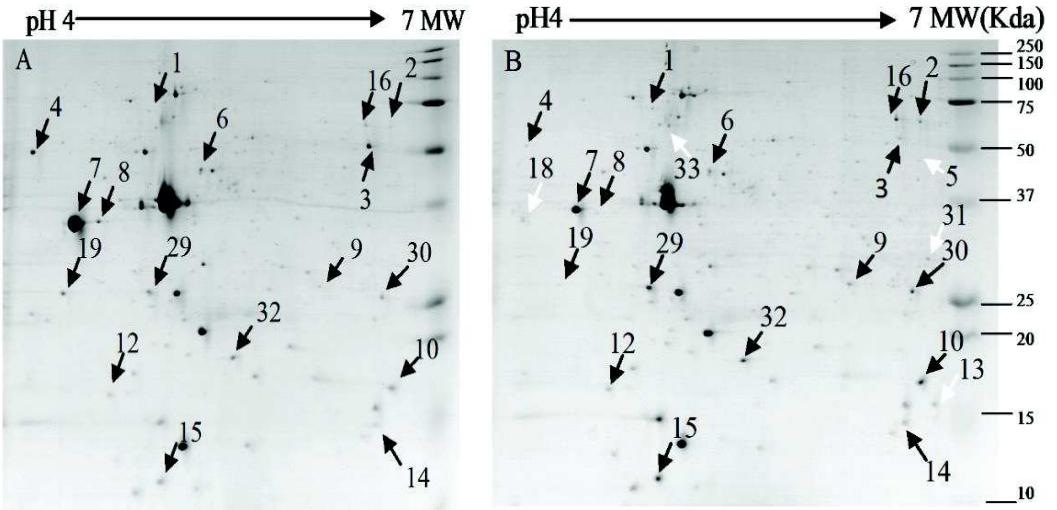

Figure 1. Two-dimensional maps (PH4-7) of Bb strain RB and HB OMPs2-DE profile of RB OMPs stained with CBB (B) 2-DE profile of HB OMPs stained with CBB

Protein identification

For protein identification, 23 protein spots were picked out from 2-DE gels and submitted to in-gel trypsin digestion for MS identification. These spots included proteins observed only in the HB gel, and proteins observed in both gels with different expression levels. The result showed that 21 spots were successfully identified, corresponding to 19 proteins. Among these proteins, five were observed only in HB (5, 13, 18, 31 and 33) , fourteen were present in both gels, but the majority showed higher expression levels in HB except five protein spots (3, 4, 7, 8 and 19). All differential protein sequences of the proteins identified were submitted to PSORT to predict protein localization and the results are shown in Table 1. Functional categories of the identified proteins of Bb were sorted using COGnitor. These identified proteins belong to different classes (Table 2).

Table 1. Protein spots identified by MALDI-TOF-MS or MALDI-TOF-TOF-MS

Spot ID	Database ID no.	Identified protein name	Theoretical MW(Da)/PI	MASCOT score	Peptides matched	Location
2,16	gi|33577555	putative outer membrane protein 1	69310.3/7.72	1,010	36	Outer Membrane
3	gi|33567775	putative outer membrane protein 2	54883.9/7.7	923	37	Outer Membrane
4	gi|33575977	flagellar hook protein FlgE	49037.9/4.28	287	8	Extracellular
8	gi|33576000	flagellar motor switch protein FliG	36594.7/4.67	755	18	Cytoplasmic
19	gi|33575979	flagellar basal-body rod protein	27716.9/4.5	636	9	Extracellular
5	gi|33577084	2-oxoglutarate dehydrogenase complex, E3 component	50357.2/6.33	173	14	Cytoplasmic
7	gi|33577259	outer membrane porin protein precursor	41278/5.51	608	16	Outer Membrane
9	gi|257696710	unnamed protein product 1	27822/	612	18	Cytoplasmic Membrane
1	gi|47115747	Chaperone protein htpG	71295.6/5.01	801	30	Cytoplasmic
6	gi|33599950	chaperonin GroEL	57447.4/5.09	287	13	Cytoplasmic
33	gi|33567547	60 kDa chaperonin	57447.4/5.09	766	25	Cytoplasmic
29	gi|33576210	putative amino acid ABC transporter solute-binding protein	28311.7/5.47	769	18	Periplasmic
30	gi|33577543	putative amino acid ABC transporter ATP-binding protein	28254.3/6.25	729	19	Cytoplasmic Membrane
31	gi|33576018	30S ribosomal protein S2	27550.2/6.4	461	16	Cytoplasmic
15	gi|33567094	putative membrane protein 1	19407.1/6.15	156	6	Unknown
32	gi|33576588	putative membrane protein 2	22788.8/5.55	787	17	Unknown
10	gi|33577008	conserved hypothetical protein 1	21632.6/6.59	678	10	Unknown
12	gi|33568581	conserved hypothetical protein 2	19819.2/5.26	656	10	Unknown
13,14	gi|33577188	putative lipoprotein	19868.9/8.74	297	9	Unknown

Table 2. The functional category of identified proteins

Functional category	The number of protein
Cell motility and secretion (N)	3
Posttranslational modification, protein turnover, chaperones (O)	3
Energy production and conversion (C)	3
Amino acid transport and metabolism (E)	1
Inorganic ion transport and metabolis (P)	1
Cell envelope biogenesis, outer membrane (M)	2
Translation, ribosomal structure and biogenesis (J)	1
Function unknown (S)	2
NO related COG[a]	3

[a] This message appears if the query protein is not predicted to belong to any of the currently-defined COGs, or if the protein is not predicted to belong to a COG composed of the minimum number of clades indicated

DISCUSSION

In our research, the profiles of OMPs of HB and RB strains were analyzed by 2-DE. The choice of the two strains was based on their virulence. HB and RB were isolated from an infectious rhinitis rabbit and a healthy rabbit, respectively. A total of 19 proteins were successfully identified by a comparative proteomics approach. In the 19 differential proteins, protein number 3, 4, 7, 8, 19 had a higher expression level in RB strain. Proteins 4, 8 and 19 were identified to be flagellin associated proteins, proteins 3 and 7 were identified to be a putative OMP and outer membrane porin protein precursor respectively. Some proteins predicted other than OMP were involved in, including extracellular, periplasmic, cytoplasmic membrane and cytoplasmic proteins. These proteins may be present in OMPs extracts (Liu et al. 2008), though sodium carbonate treatment had a higher rate of OMPs extraction (Suh et al. 2008).

The research results showed that flagellin-associated proteins were both indentified in the two strains and RB strain had a higher expression of these proteins. Infection with a Bb strain which expresses flagellin could result in a strong antibody response to flagellin and reduce the colonization of Bb in the trachea, which indicates that flagellin repression is important for *Bordetella* sp. Infection (Akerley et al. 1995). A 40kD outer membrane porin is present in both the virulent strain and avirulent strain of Bb. The expression of this protein had a higher level in RB than in HB strains in our research. This protein usually forms anion-selective, small channels in lipid bilayer membranes. The other 14 proteins had a higher expression level in HB strain, in which, four proteins (5, 13, 31, 33) were proved to exist only in the HB strain. Protein 5 was identified to be 2-oxoglutarate dehydrogenase complex, E3 component. It is an important protein in the tricarboxylic acid cycle. Protein 31 was identified to be the 30S ribosomal protein S2. The 30S ribosomal subunit and tRNA binding is a key step in protein synthesis. These two proteins are predicted to locate in the cytoplasm, relate to energy production and conversion. In our study, three proteins were identified to be chaperones, which belong to a member of the heat shock protein family. Protein 1 was identified to be chaperone protein htpG, belonging to the heat shock protein90 (HSP90) family members. Protein 6 was identified to be chaperonin GroEL, and protein 33 was identified to be 60KD chaperonin. These two proteins both belong to the heat shock protein HSP60 family members. Chaperones play an important role in the polypeptide folding process. Hsp60 is displayed on the surface of bacteria, and is universally expressed in inflammation. Hsp60 is well recognized by the immune system and can affect the innate and acquired immune systems to generate humoral and cellular immunity. The expression of HSP90 doubles quickly to protect the cells against endogenous attack, enhance cell repair and improve the

tolerance of cells against stimulation. GroEL can influence probiotic properties of some bacterium, including biofilm formation, tolerance to acid, etc (Lemos et al. 2007).

Seven putative proteins and two hypothetical proteins were identified in our research. Some of them were not related to OMPs, by the predicted cellular location analysis, but they may be related to the virulence or phenotypic features because that they are located in membrane, and may be the first step in host interaction (Taddei et al. 2011). For example, putative amino acid ABC transporter which had a higher expression level in the virulent strain HB, also had a higher expression level in Mycobacterium.

In conclusion, 19 proteins were identified by comparative proteome analysis of OMPs of two different virulent Bb strains. These proteins may be associated with virulence of Bb, however these findings still need further studies.

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