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Proteomic analysis of *Helicobacter pylori* J99 Outer Membrane Protein by Tandem Mass Spectrometry

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The protein identity of sarcosine-insoluble outer membrane proteins (OMPs) of *Helicobacter pylori* J99 was determined with the basic study of understanding the function of proteins. A sarcosine-insoluble OMPs was resolved by two-dimensional electrophoresis with immobilized pH gradient strips. The most abundant proteins were shown in the alkaline p*I* regions (6.0~11.0) with molecular masses of 10 to 100 kDa. We have performed an extensive proteome analysis by quadrupole time of flight (Q-TOF) mass spectrometry (MS). Here, of 50 spots processed, 42 spots were identified, which represented 16 genes and we newly detected 8 kinds of proteins (JHP0119, JHP0388, JHP1046, JHP1405, JHP0073, JHP0551, JHP1382, JHP0552) from the sarcosin-insoluble fraction of *H. pylori* J99. Those may be used to elucidate the characterization of the OMPs of *H. pylori* J99, which will help identify new potential target proteins for vaccine development and drug therapy.

Key Words: *Helicobacter pylori*, Outer membrane protein, Q-TOF MS

INTRODUCTION

Helicobacter pylori is a microaerophilic and gram-negative spiral bacterium. Since Warran and Marshell isolated bacteria successfully in 1983, it has been identified as the major pathogen of gastroduodenal disease that is mediated in part by its outer membrane proteins (OMPs) (4) including BabA (9), AlpA/AlpB (16), HopZ (17), and SabA (12). These proteins are known to adhere to gastric epithelial cells as adhesins. Moreover, porins determine the permeability properties of the outer membrane and are also immunologically active, can act as protective antigens which often represent the most significant antigenic determinants of a particular bacterial species (8).

The whole genome sequence of *H. pylori* J99 has been reported (1). The sequence has provided enormous insights into the biology of this organism and made possible detailed studies of this bacterium. The genome of *H. pylori* J99 consists of 1495 predicted genes, making it accessible to proteome analysis. Our proteome analysis relied on the separation of proteins by two-dimensional electrophoresis (2-DE), peptide fingerprinting by quadrupole time of flight-

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mass spectrometry (Q-TOF-MS), and protein identification by searching gene databases. Proteome analysis is required to understand and validate the results of genomic sequence analysis by determining the authenticity of theoretical open reading frames, quantifying gene expression, and identifying post-translational modifications of gene products.

In the present study, we have the 2-DE profiles of the sarcosine-insoluble proteins to provide the guidance for *H. pylori* J99 OMPs by Q-TOF-MS. Out of 61 theoretically predicted OMPs, we identified 42 spots and newly detected 8 kinds of protein compared to *H. pylori* 26695.

MATERIALS AND METHODS

1. Bacterial strain and culture conditions

H. pylori strain J99 was incubated onto a brucella agar plate containing 10% bovine serum, vancomycin (6.9 μM), and amphotericin B (1.1 μM). Bacterial cells were cultivated overnight at 37℃ under 10% CO_2 and 100% humid atmosphere.

2. Preparation of Sarcosine-insoluble OMPs

The sarcosine-insoluble fraction of *H. pylori* J99 was prepared as previously described (2). The harvested cells were suspended in 20 mM Tris-HCl (pH 7.5) and disrupted with an ultrasonicator (Sonics & Materials Inc. Danbury, CT, USA). The precipitate was collected by centrifugation (40,000 X g, 30 min, 4℃) and resuspended in 20 mM Tris-HCl (pH 7.5) containing 2.0% (wt/vol) sodium lauryl sarcosine. The sarcosine insoluble fraction was collected by centrifugation (40,000 X g, 30 min, 4℃) and washed three times with distilled water.

3. Two-dimensional electrophoresis and Peptide clean-up for Nanoelectrospray MS/MS

Isoelectric focusing (IEF) was performed using immobilized pH gradient (IPG) strips with a pH range of 6.0~11.0. (17 cm, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After IEF, two-dimensional electrophoresis and silver staining were processed as previously described (2). After in-gel digestion with trypsin, the peptide solution was passed through a GELoader tip (Eppendorf) packed with a poros R2 resin (PerSeptive Biosystems, Framingham, MA, USA) washed twice with 20 μl of 5% methanol/3% formic acid, and eluted with 2.5 μl of 70% methanol/3% formic acid. To retain the column resin, the end of the GELoader tip was constricted by pressing it with a pair of tweezers. A 1 ml syringe was used to force liquid through the column by applying gentle air pressure (3).

4. Nanoelectrospray MS/MS

MS/MS data was obtained using a QSTAR pulsar-i mass spectrometry system (AB/MDS Sciex, Toronto, Canada) equipped with a nanoelectrospray ion source (MDS protana, Odense, Denmark). Ionspray voltage was set to a potential of 900~1000V. Scan data for the tryptic peptides was acquired over the m/z range 400~1600 Da in positive mode. MS/MS experiments were performed over the m/z range 80~1600 or 80~2000 Da with manually optimized collision energy settings for each peptide. The data was processed and interpreted with the software, BioAnalyst (PerSeptive Biosystems, Foster City, CA, USA). The resulting peptide sequence tags were used for a homology search of the NCBInr database using Mascot software (Matrix Science Ltd., London, UK) for protein identification. Mascot MS/MS ion search criteria were as follows: taxonomy-all entries, trypsin digestion allowing up to one miss cleavage, peptide tolerance of 2.0 Da, and MS/MS tolerance of 0.8 Da. The "ion score cut-off" was manually set to 20 thereby eliminating the lowest quality matches. A probability based Mowse score>28 indicated identity ($p<0.05$).

RESULT

1. Two-DE of the sarcosine-insoluble fraction of *H. pylori* J99

We report the proteome analysis of the *H. pylori* J99 sarcosine insoluble fraction by Q-TOF-MS. Isoelectric focusing (IEF) was performed using immobilized pH gradient (IPG) strips with a pH range of 6.0~11.0. Fig. 1 shows that sarcosine-insoluble proteins were enriched in the alkaline pI region of the 2-DE gel and that their mole-

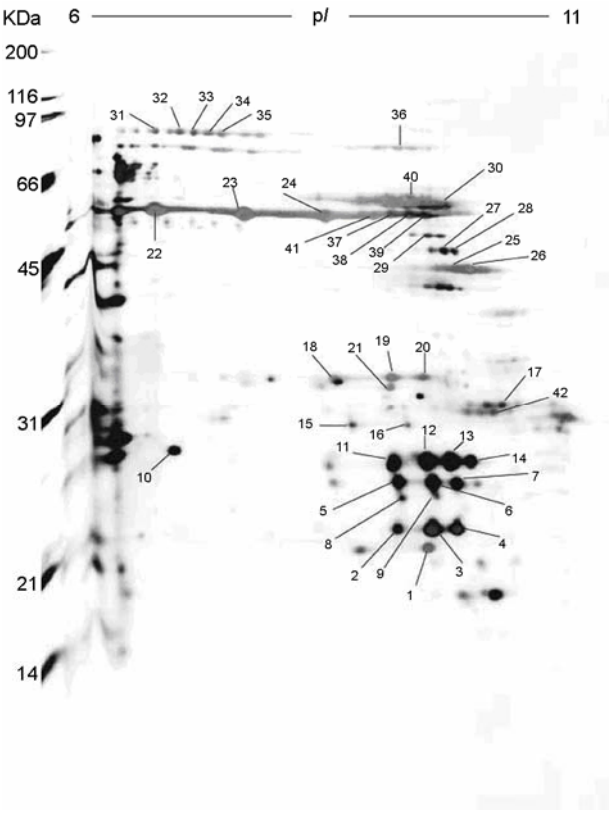

Figure 1. 2-DE of sarcosine-insoluble outer membrane fraction of *H. pylori* J99 with an IPG strip, pH 6.0 to11.0. Spots that were analyzed by Q-TOF MS are numbered.

cular masses were between approximately 10 and 100 KDa. The theoretical or observed pI values of the majority of the OMPs identified in this study were above 9.0. As shown in Fig. 1, the silver stained spots generated by 2-DE were numbered, excised, destained, and followed by in-gel digestion using trypsin for peptide fingerprinting. Among the all represented proteins spots processed, about 50 spots were visible on 2-DE gel, as shown in Fig. 1, 42 spots including newly 8 kinds of protein were identified, which represented 16 genes. The database was searched to determine the sequence identity of these spots.

2. Nanoelectrospray MS/MS and protein identification

As shown in Fig. 1, the silver stained spots generated by 2-DE were numbered, excised, destained, and followed by in-gel digestion using trypsin for analysis by a QSTAR pulsar-i mass spectrometry system equipped with a nano-electrospray ion source. Among the all represented proteins spots processed, about 50 spots were visible on 2-DE gel, as shown in Fig. 1, 42 spots including newly 8 kinds of protein were identified, which represented 16 genes (Table 1). The database was searched to determine the sequence identity of these spots. Among the 42 spots, horizontally located spots were identified as a putative protein (JHP1100, spot no 2, 3, 4), OMP-porin JHP0849 (spot no 22, 23, 24, 41) and JHP1405 (spot no 31, 32, 33, 34, 35). JHP1405 was identified as a putative iron regulated OMP. Nine spots on 2-DE gel (spot no 5, 6, 7, 8, 9, 11, 12, 13, 14) all turned out to be the hypothetical protein JHP 0119. OMP-adhesin JHP0833 (spot no 15, 16), OMP-porin JHP0645 (spot no 18, 19, 20), JHP1394 (spot no 25, 26, 27, 28) and hypothetical protein JHP0552 (spot no 37, 38, 39) gave similar horizontal spot arrays. Spot number 10 was identified as gamma glutamyl transpeptidase (GGT, JHP1046). We newly identified 8 kinds of proteins (JHP0119, JHP0388, JHP1046, JHP1405, JHP0073, JHP0551, JHP1382, JHP0552) from the sarcosin-insoluble fraction of *H. pylori* J99.

DISCUSSION

In our previous study, we identified the sarcosine-insoluble fraction of *H. pylori* 26695 by MALDI-TOF-MS. Here, we report the proteome analysis of the *H. pylori* J99 sarcosine insoluble fraction by Q-TOF-MS, which allowed us make a comparative analysis of the proteome maps of both strains.

Fig. 1 shows that sarcosine-insoluble proteins were enriched in the alkaline pI region of the 2-DE gel, which is higher than that of other bacterial OMPs. In contrast to *H. pylori* OMPs, it has been reported that the pI ranges of OMPs of *Escherichia coli* (14), *Salmonella enterica* serovar Typhimurium (15), *Klebsiella pneumoniae* (15), *Caulobacter crescentus* (15), and *Leptospira interrogans* serovar Lai (6) are situated between pI 4 to 7. This may reflect evolutionary pressure for high alkaline proteins because of the acidic environment of *H. pylori*.

About 50 spots were visible on 2-DE gel, as shown in Fig. 1, 42 spots were identified. However some spots did not produce spectra in Q-TOF-MS, even though their inten-

Table 1. List of identified proteins in the sarcosine-insoluble outer membrane fraction of *H. pylori* J99

Spot no	Protein identification	Accession no[a]	No. of peptide matches	Score[b] and seq. C (%)[c]	Amino acid sequence	Tp*I*[d]	TM*r*[d]	JHP no[a]
1	Putative outer membrane protein	gi|15611491	1	37, 5.4	FQFLWNLGGR	9.39	20895	JHP0424
2	Hypothetical protein	gi|15612165	1	36, 4.7	FSYEDSLLK	9.46	20838	JHP1100
3	Hypothetical protein	gi|15612165	1	38, 5.9	SFIDGDLDIQK	9.46	20838	JHP1100
4	Hypothetical protein	gi|15612165	1	89, 15.1	GQVITLIGQNEVPYLILETDCQVGDIAK	9.46	20838	JHP1100
5	Hypothetical protein	gi|15611189	1	61, 3.9	GDLSAFGAFFK	9.93	32642	JHP0119
6	Hypothetical protein	gi|15611189	1	47, 4.6	K.GDLSAFGAFFK.G	9.93	32642	JHP0119
7	Hypothetical protein	gi|15611189	1	30, 4.6	K.GDLSAFGAFFK.G	9.93	32642	JHP0119
8	Hypothetical protein	gi|15611189	1	20, 4.6	K.GDLSAFGAFFK.G	9.93	32642	JHP0119
9	Hypothetical protein	gi|15611189	1	35, 4.6	K.GDLSAFGAFFK.G	9.93	32642	JHP0119
10	Gamma-glutamyltranspeptidase (ggt)	gi|15612111	1	32, 2.1	VFLVVGSPGGSR	9.72	61089	JHP1046
11	Hypothetical protein	gi|15611189	3	135, 14.4	K.GDLSAFGAFFK.G K.VSFVVNDR.E K.DLGTELSLPLFNWLYK.G	9.93	32642	JHP0119
12	Hypothetical protein	gi|15611189	2	79, 10.9	GDLSAFGAFFK GSDFGALHEQFGDMYDGYIK	9.93	32642	JHP0119
13	Hypothetical protein	gi|15611189	1	23, 7.0	GSDFGALHEQFGDMYDGYIK	9.93	32642	JHP0119
14	Hypothetical protein	gi|15611189	1	41, 3.9	GDLSAFGAFFK	9.93	32642	JHP0119
15	Outer membrane protein-adhesin (babA)	gi|15611900	3	132, 5.2	YSTLNTLIK LSADPSAINAVR GIQDLSDRYESLNNLLNR	9.16	80607	JHP0833
16	Outer membrane protein-adhesin (babA)	gi|15611900	2	88, 2.8	LSADPSAINAVR YSTLNTLIK	9.16	80607	JHP0833
17	Putative outer membrane protein	gi|15612427	2	47, 9.7	VYAFQISYLR FPPYAGPGFEVGYK	9.90	28238	JHP1362

Table 1. Continued

18	Outer membrane protein-porin (hopE)	gi|15611712	2	63, 13.3	K.YANGALNGFGLNVGYK.K K.FLSAGPNATNLYYHLK.R	9.11	29551	JHP0645
19	Outer membrane protein-porin (hopE)	gi|15611712	1	30, 5.9	FLSAGPNATNLYYHLK	9.11	29551	JHP0645
20	Outer membrane protein-porin (hopE)	gi|15611712	1	40, 5.9	FLSAGPNATNLYYHLK	9.11	29551	JHP0645
21	Putative outer membrane protein	gi|15611144	2	72, 9.8	R.GVDGSVDVFYK.R K.LPLFTNQFYK.E	9.05	28351	JHP0073
22	Outer membrane protein/porin (hopB)	gi|15611916	3	100, 12	K.FQFLFDVGLR.M K.SHNQHSIEIGVQIPTIYNTYYK.A K.ANPWLGNFAAGNSSQVNAFNGFITK.I	9.33	56753	JHP0849
23	Outer membrane protein/porin (hopB)	gi|15611916	1	52, 2.3	K.FQFLFDVGLR.M	9.33	56753	JHP0849
24	Outer membrane protein/porin (hopB)	gi|15611916	2	76, 7.4	K.FQFLFDVGLR.M K.ANPWLGNFAAGNSSQVNAFNGFITK.I	9.33	56753	JHP0849
25	Putative outer membrane protein	gi|15612459	1	45, 2.6	IPTLPNYFFK	9.75	42902	JHP1394
26	Putative outer membrane protein	gi|15612459	2	78, 9.3	IPTLPNYFFK QGPLENGNPTTITGAETNFSLTQTLR	9.75	42902	JHP1394
27	Putative outer membrane protein	gi|15612459	1	26, 3.1	K.IPTLPNYFFK.G	9.75	42902	JHP1394
28	Putative outer membrane protein	gi|15612459	1	54, 3.1	K.IPTLPNYFFK.G	9.75	42902	JHP1394
29	Hypothetical protein	gi|15611619	2	87, 5.7	K.TTIDAPNLQLR.E R.LTLEYLTNLSVK.N	9.39	54624	JHP0552
30	Putative	gi|15612447	2	73, 5.0	R.NTLSSIIIVEQK.S K.SLLSSVELAK.E	9.51	56924	JHP1382
31	Putative iron regulated outer membrane protein (frpB_3)	gi|15612470	3	100, 5.6	R.VESTAFLGVR.G R.YDIYTLLDK.N R.THVTSGFSPSATVLYNPIESIGLK.V	9.16	97572	JHP1405

Table 1. Continued

32	Putative iron regulated outer membrane protein (frpB_3)	gi|15612470	2	77, 2.8	R.VESTAFLGVR.G R.IFLINSGVNVK.V	9.16	97572	JHP1405
33	Putative iron regulated outer membrane protein (frpB_3)	gi|15612470	2	71, 2.6	R.VESTAFLGVR.G R.YDIYTLLDK.N	9.16	97572	JHP1405
34	Putative iron regulated outer membrane protein (frpB_3)	gi|15612470	3	108, 4.1	R.VESTAFLGVR.G R.IFLINSGVNVK.V R.YDIYTLLDK.N	9.16	97572	JHP1405
35	Putative iron regulated outer membrane protein (frpB_3)	gi|15612470	3	108, 4.1	R.VESTAFLGVR.G R.YDIYTLLDK.N R.IFLINSGVNVK.V	9.16	97572	JHP1405
36	Putative outer membrane function	gi|15612168	1	47, 2.3	IPTINTNYYSYLGTK	9.51	69547	JHP1103
37	Hypothetical protein	gi|15611619	2	42, 5.2	K.AALGLYELLK.G K.TTIDAPNLQLR.E	9.39	54624	JHP0552
38	Hypothetical protein	gi|15611619	4	121, 11.5	K.AALGLYELLK.G K.TTIDAPNLQLR.E R.LTLEYLTNLSVK.N K.ASLDAANLSFANIK.R	9.39	54624	JHP0552
39	Hypothetical protein	gi|15611619	2	47, 5.2	K.AALGLYELLK.G K.TTIDAPNLQLR.E	9.39	54624	JHP0552
40	Outer membrane protein/porin (hopC)	gi|15611915	1	20, 2.5	R.ATNILNGFYTK.V	9.36	56404	JHP0848
41	Outer membrane protein/porin (hopB)		2	104, 5.3	K.FQFLFDVGLR.M R.STQLLNNTTNTLAK.V	9.33	56753	JHP0849
42	Putative outer membrane protein	gi|15612326	1	54, 1.9	YNQLQTVAQELGK	6.52	75659	JHP1261

[a] accession number and JHP number are obtained from NCBI database; [b] score, probability score in Mascot program; [c] seq. C (%), percentage of sequence coverage; [d] Tp*I* and T M*r*, Theoretical p*I* and M*r* are from www.tigr.org.

sities were quite high on the gels. From the Q-TOF-MS spectrum of each individual spot, 15~20 peptide peaks, whose count intensities were 4-fold higher than that of background noise, were used to search the database.

Among the 42 spots, three horizontally located spots (spot no 2, 3, 4) were identified as a putative protein (JHP1100) that is homologous to HP1173 in *H. pylori* 26695. HP1173 is secreted into the extracellular medium (5) and was previously identified as an immunoreactive protein (2). However, the function of this protein is not known. Some proteins, such as OMP-porin JHP0849 (spot no 22, 23, 24, 41) and JHP1405 (spot no 31, 32, 33, 34, 35) produced several horizontally aligned spots. JHP1405 was identified as a putative iron regulated OMP, which was only detected in *H. pylori* J99 but *H. pylori* 26695. It was previously designated as frpB, an iron-regulated protein, located in the outer membrane. It acts as an enterobactin receptor in *Neisseria gonorrhoeae* (7) and as a ferric citrate receptor in *E. coli*.

Nine spots on 2-DE gel (spot no 5, 6, 7, 8, 9, 11, 12, 13, 14) all turned out to be the hypothetical protein JHP 0119, which is truly novel protein compared to *H. pylori* 26695 in 2-DE map. Their estimated molecular weights and pI values varied, since the predicted value of this hypothetical protein (JHP0119) is 32.6 KDa with a pI 9.93. The molecular weights and pI values of the protein spots on 2-DE gels were estimated and compared to their theoretical values derived from the genome sequence. This protein is only detected in *H. pylori* J99 and maybe used to characterization of the OMPs of *H. pylori* J99.

Some protein spots migrated on the gels to molecular weights that were different from those calculated from the genome sequences. These mismatches might be caused by random proteolysis during fractionation. OMP-adhesin JHP-0833 (spot no 15, 16), OMP-porin JHP0645 (spot no 18, 19, 20), JHP1394 (spot no 25, 26, 27, 28), and hypothetical protein JHP0552 (spot no 37, 38, 39) gave similar horizontal spot arrays. Alternatively, this pattern may be due to post-translational modifications, which would result in differentially charged side chains on the amino acids residues, resulting in differential pI values on the gels (11). Of the other proteins identified, the putative OMP JHP0424 is homologous to OMP11 in *H. pylori* 26695. JHP1100 and JHP0424 are homologous to HP1173 and HP0472 respectively, and are immunoreactive proteins (2). The putative OMPs encoded by jhp1362, jhp1394, jhp0645, jhp0073, jhp1261 and jhp1103 are homologous to omp31, omp32, omp15, omp3, omp29 and omp27 of *H. pylori* 26695, respectively.

Spot number 10 was identified as gamma glutamyl transpeptidase (GGT, JHP1046), a protein of 61 KDa, although the observed value (38 KDa) was smaller than expected. Mature GGT is a heterodimer (38 KDa + 20 KDa) and a periplasmic enzyme, suggesting that the spot may be the large subunit of this periplasmic protein (13).

The functions of at least five proteins have been already predicted. A family of five OMPs from *H. pylori*, termed HopA to HopE, share N-terminal sequence homology and have been shown to function as porins (8). OMP-adhesin JHP0833 encoded by omp28 is a homologue of BabA, which is a Lewie B binding adhesin (16). JHP0645, JHP-0849, and JHP0848 have previously been designated HopE, HopB, and HopC, respectively. We were able to predict that these proteins would function as porins. Also, JHP-0849 (HopB) and JHP0848 (HopC) have been reported to be enriched in the supernatants of *H. pylori* was grown in the absence of nalidixic acid (10).

We newly identified 8 kinds of proteins (JHP0119, JHP-0388, JHP1046, JHP1405, JHP0073, JHP0551, JHP1382, JHP0552) from the sarcosin-insoluble fraction of *H. pylori* J99. Those may be used to elucidate the biological function and the characterization of the OMPs of *H. pylori* J99, which will help identify new potential target proteins for vaccine development and drug therapy.

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