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Original Article

Identification of *Helicobacter pylori* Strain 51 Major Outer Membrane Proteins by Quadrupole Time of Flight Mass Spectrometry

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As part of an initial inquiry into the function of the outer membrane proteins (OMPs) of *Helicobacter pylori* Korean strain 51, we have conducted an extensive proteome analysis via quadrupole time of flight (Q-TOF) mass spectrometry (MS). Fifty one OMPs of *H. pylori* were purified using sarcosine and resolved via two-dimensional electrophoresis with immobilized pH gradient strips. The most abundant proteins were observed in the alkaline p*I* regions (6.0~11.0) at molecular masses between 10~100 KDa. Here, 15 spots were identified, representing 9 types of genes (KHP0852, KHP0853, KHP1353, KHP1017, KHP0172, KHP0076, KHP0617, KHP1069, KHP0614) from the sarcosin-insoluble fraction of *H. pylori* 51. These may be employed in the characterization of the OMPs of *H. pylori* 51, which will help to identify new potential target proteins for vaccine development and drug therapy.

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

INTRODUCTION

The whole genome sequence of *Helicobacter pylori* strain 51 (Korean isolate) has been previously reported (1). Far Eastern countries, including Korea, Japan, and China, demonstrate the highest worldwide prevalence of *H. pylori* infection. Consequently, these countries also have the highest incidence of gastroduodenal disorders, including gastric cancers (2~4). In the gram-negative bacteria, many surfaceexposed proteins are constituents of the outer membrane (OM), which forms a barrier between the cell and its surroundings. The OM performs a structural function, and also performs a major role in determining what enters the cell, what molecules are secreted from the cell, and how the cell interacts with its environment. The OM is rich in transmembrane proteins, the functions of which include motility in transporting nutrients to and from the cells, conjugation, and the control of cell morphology (5). Interest has been increasing in the role of *H. pylori* outer membrane proteins (OMPs) in gastroduodenal diseases, and new findings have been reported in relation to OMPs and clinical outcomes and gastric histology (6). However, little is currently known concerning the OMPs of *H. pylori* or the identities of them. Only a few proteins have been identified thus far as being unambiguously localized within the OM (7, 8). In an effort to confirm the predicted surface proteins and to identify additional candidates, proteome analysis is a particularly appropriate method. The OM of *H.*

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pylori is quite difficult to isolate without inner membrane contamination, and appears to be strongly associated with the inner membrane. In order to enrich surface proteins for proteome analysis and to avoid potential problems with contamination from the inner membrane and the cytoplasm, *H. pylori* was treated with sarcosine (9) and subsequently analyzed via 2-DE, via which potential surface proteins were detected. These findings could contribute to new proteome analysis applications and to the construction of the OMP 2-DE map of the *H. pylori* strain.

In this study, on the basis of the genomic sequencing information for *H. pylori* OMP annotation, the major proteome components of *H. pylori* strain 51 were analyzed. Our findings may prove useful in elucidating the biological functions of the Korean strain *H. pylori* 51, and would therefore also prove useful in diagnostics and vaccine development.

MATERIALS & METHODS

Bacterial strain and growth condition

H. pylori strain 51 was isolated from a Korean patient with a duodenal ulcer. *H. pylori* strain 51 was stored in a liquid nitrogen tank, and then thawed and incubated onto a brucella agar plate containing 10% bovine serum, vancomycin (6.9 μM), and amphotericin B (1.1 μM). The bacterial cells were cultivated overnight at 37℃ under 10% CO_2 and a 100% humid atmosphere. The bacteria were washed in washing solution (40 mM Tris-HCl, pH 7.2, 1 mM EDTA) twice via centrifugation. *H. pylori* strain 51 was deposited in and supplied from the *H. pylori* Korean Type Culture Collection (Gyeongsang National University School of Medicine, Jinju, Korea).

Sarcosine preparation of *H. pylori* OMPs

The sarcosine-insoluble outer membrane fraction of *H. pylori* was prepared as described previously (9) with minor modifications. *H. pylori* cells were harvested via centrifugation (12,000 × g, 20 min, 4℃) and washed three times with 20 mM Tris-HCl, pH 7.5. The cells were then suspended in 20 mM Tris-HCl, pH 7.5, and disrupted with an ultrasonicator (Sonics & Materials Inc. Danbury, CT, USA). DNase and RNase (20 g/ml each) were added to the cell suspension, and the mixture was incubated for 30 min at room temperature. Total membrane proteins were then collected via centrifugation (40,000 × g, 30 min, 4℃). The membranes were resuspended in 20 mM Tris-HCl, pH 7.5 containing 2.0% (w/v) sodium lauryl sarcosine, and incubated for 30 min at room temperature. The OMPs were collected via centrifugation (40,000 × g, 30 min, 4℃) and washed three times in DW. The pellets were then resuspended in DW, aliquoted, and stored at -20℃ until use.

Two-dimensional gel electrophoresis (2-DE)

Isoelectric focusing (IEF) was conducted using IPG strips (Bio-Rad, Hercules, CA, USA). Portions (300 μg) of the OMPs were applied to pH strips in ranges of 3.0 to 10.0 and 6.0 to 11.0. The samples were diluted via incubation in a rehydration solution containing 7 M urea, 2 M thiourea, 2 mM tributyl phosphine (Sigma-Aldrich, St. Louis, MO, USA), 40 mM Tris base, 1% Triton X-100, and 0.5% ampholyte (pH 3.0 to 10.0 [Bio-Rad] and pH 6.0 to 11.0 [Amersham, Little Chalfont, Bucks, UK]) overnight in a reswelling tray (Bio-Rad). The strips were rehydrated under the following passive conditions: 0 V, 20℃, and a 14- to 16-h reaction time in a Protean IEF cell (Bio-Rad). Three preset programs were executed with slight modifications, such that the focusing conditions comprised the conditioning step, voltage ramping, and final focusing. After IEF, the strips were equilibrated in 0.375 M Tris buffer (pH 8.8) containing 6 M urea, 2% SDS, 20% glycerol, 2% dithiothreitol, and 0.01% bromophenol blue, followed by the addition of the same buffer supplemented with 2.5% iodoacetamide. Separation in the second dimension was conducted at 30 mA/gel at 4℃, until the running dye reached the bottom.

Silver staining

Silver staining was followed by the method developed by Heukeshoven *et al* (10) with slight modifications. In brief, the gels were fixed for 90 min with fixing solution containing 50% methanol, 12% acetic acid, and 0.5 ml

37% formaldehyde/L. All incubation was conducted in a shaker with gentle shaking. After fixing, the gels were washed twice for 20 min each with 50% ethanol, and then washed for an additional 20 min with dDW. The gels were pre-treated for 1 min with $Na_2S_2 \cdot 5H_2O$ (0.1 g/L) and again washed with dDW. Silver nitrate was impregnated via 30 min of incubation in $AgNO_3$ (2 g/L) and 0.75 ml 37% HCOH/L, and then rinsed three times with dDW for 20 sec each. Visualization was conducted by incubation in developing solution until a clear image emerged. The solution consisted of Na_2CO_3 (60 g/L), $Na_2S_2 \cdot 5H_2O$ (0.2 g/L) and 0.5 ml 37% HCOH, which was prepared previously and preserved in an ice slurry. When clear spots emerged, the gel was washed twice with dDW for 20 sec each, and then the reaction was halted via the addition of 50% MetOH and 12% AcOH for 10 min.

Gel images were obtained by scanning the silver-stained gels with Fluor-S MultiImages (Bio-Rad) and documented with the PDQUEST program on a Samsung Magic Station M 5660.

Spot processing for nanoelectrospray MS/MS

The silver-stained proteins were destained with the chemical reducers to remove the silver and tryptic digestion was conducted via the method previously described by O'Connell & Stults (11, 12). After overnight digestion at 37℃, 20 μl of the digested solution was dried for 1 h in a vacuum centrifuge. Dried samples were dissolved with 2 μl of 0.1% TFA (trifluoroacetic acid; Aldrich). The remaining tryptic digestion supernatant was passed through a microcolumn packed with a porous R2 resin (PerSeptive Biosystems, Framingham, MA, USA) in GELoader tips (Eppendorf, Hamburg, Germany), 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 with a pair of tweezers. A 1 ml syringe was used to force liquid through the column via the application of gentle air pressure (13).

MS/MS data was acquired with a QSTAR pulsar-i mass spectrometry system (AB/MDS Sciex, Toronto, Canada) equipped with a nanoelectrospray ion source (MDS Protana, Odense, Denmark). Two microliters of peptide solution were loaded into a long spray capillary tube (MDS Protana) and the ends of the tubes were opened by touching them against a surface in the source. The ionspray voltage was set to a potential of 850-900V. The tryptic peptides were acquired over the m/z range of 400~1600 Da in the positive mode. MS/MS experiments were conducted over the m/z range of 80~1600 or 80~2000 Da with manually optimized collision energy settings for each peptide. The data were processed and interpreted using BioAnalyst software (PerSeptive Biosystems).

The resultant peptide sequence was submitted to proteinInfo (http://service.proteometrics.com/PROWL/Proteininfo.html) against the NCBInr database for identification. MASCOT™ (http://www.matrixscience.com) was used to search and interpret the raw MS/MS data. When searching the product ion spectra, a mass accuracy of ±0.5 Da in the masses of both precursor and product ions was selected.

RESULT

We performed proteome analysis of *H. pylori* strain 51 via nanoelectrospray MS/MS. The sarcosine-insoluble protein solution of the strain was loaded onto IPG strips with a pH gradient ranging from 6.0 to 11.0 for the first-dimension separation. The strips were then loaded onto 12.5% acrylamide gel for the second-dimensional electrophoresis. After running SDS-PAGE, the separated spots were visualized via silver staining. The most abundant protein spots were enriched in the alkaline p*I* regions, and their molecular weights were measured in a range between approximately 10~100 kDa.

As is shown in Figure 1, the silver-stained spots generated by 2-DE using the IPG strips of pH 6.0~11.0 were numbered, excised, and destained, followed by in-gel digestion with trypsin for peptide fingerprinting. The masses of the resultant peptide mixtures were measured via Q-TOF-MS. Among the protein spots processed, 15 were identified as significant, including the putative OMPs, KHP0852, KHP0853, KHP1353, KHP1017, KHP0172, KHP0076, KHP-

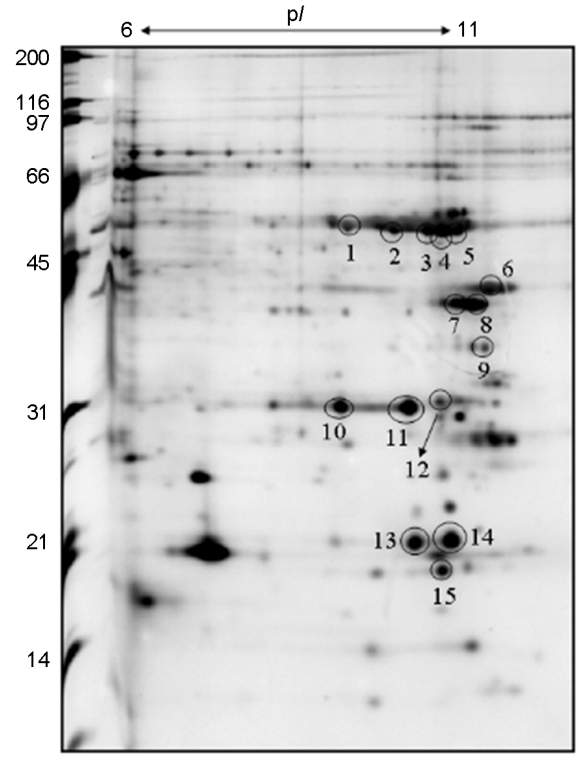

Figure 1. *H. pylori* strain 51 outer membrane protein 2-DE map. The sarcosyl-insoluble proteins were separated on an IPG strip and detected via silver staining. The proteins of the circled spots were identified by Q-TOF.

0617, KHP1069, and KHP0614, as shown in Table 1. The identified spots corresponded to 9 genes. The gel-estimated p*I* values corresponded strongly to the theoretical values of the protein spots, whose p*I* values were alkaline. However, some protein spots exhibited slight variations between the gel-estimated values and the calculated ones.

DISCUSSION

The proteome analysis technique has developed as the result of the broad availability of whole genome sequences of a number of bacterial species (14, 15). It has also been suggested that new targets must be explored for the further development of diagnosis, treatment, and prevention of *H. pylori* infections. Proteome analysis could prove an effective complement for genome-based investigation techniques for the determination of bacterial targets, and may also provide with a better understanding of the holistic biology of *H. pylori*.

A Q-TOF mass spectrometer was used to identify proteins separated by one-dimensional or two-dimensional gel electrophoresis at the femtomolar level. The high mass

Table 1. Identification of *H. pylori* 51 OMPs by amino acid sequence analysis

Spot no	Protein name	Mass (KDa)	p*I*	Amino acid sequence	KHP NO.
1	Outer rmembrane protein (OMP21)	57,027	9.8	FQFLFDVGLR	KHP0853
2	Outer membrane protein (OMP21)	57,027	9.8	STQLLNNTTNTLAK	KHP0853
3	Outer membrane protein (OMP20)	55,897	9.4	QDELLLEAFNSAVAANI	KHP0852
4	Outer membrane protein (OMP21)	57,027	9.8	STQLLNNTTNTLAK	KHP0853
5	Outer membrane protein (OMP21)	57,027	9.8	FQFLFDVGLR	KHP0853
6	Outer membrane protein (OMP32)	42,948	9.8	IPTLPNYFFK	KHP1353
7	Gamma-glutamyltranspeptidase(GGT)	61,113	10.1	VGLALSSHPLASEIGQK	KHP1017
8	Gamma-glutamyltranspeptidase(GGT)	61,113	10.1	GFYQGQVAELT	KHP1017
9	Cell binding factor 2	34,010	10.1	AAFALTPGDYTK	KHP0172
10	Urease protein Ure A	26,613	9.0	SVELIDIGGNR	KHP0076
11	Urease protein Ure A	26,655	8.43	IFGFNALVDR	KHP0076
12	Outer membrane protein	30,157	9.47	YLGTNYQLGQA	KHP0617
13	Hypothetical protein HP1173	20,747	9.46	ISLVGNFDGTGFLTEYK	KHP1069
14	Hypothetical protein HP1173	20,747	9.46	ISLVGNFDGTGFLTEYK	KHP1069
15	Hypothetical protein	32,818	10	GVAFSVDSNLK	KHP0614

resolution and high mass accuracy of this instrument in MS/MS mode allows for the identification of a protein by tandem mass spectra acquired by collision-induced dissociation of individual peptide precursors (16, 17).

This approach has proven to be useful in the comprehensive characterization of protein in-gel digests, in the dissection of complex protein mixtures, and in the sequencing of a low-molecular weight integral membrane protein. Proteins can be identified in all types of sequence databases, including an EST database. Thus, Q-TOF mass spectrometry may be a method with a remarkable potential for advancing proteomic research.

The proteome analysis of the *H. pylori* strain 26695 OMPs was reported recently (16). This study discussed the identification of 62 proteins from *H. pylori* strain 26695. Our results addressed the homology of the *H. pylori* strain 26695 proteins addressed in the previous study. We identified an additional 13 protein spots, which could contribute to the extension of proteome analysis and might also help to elucidate their potential roles in the pathogenesis of this strain.

It has been previously suggested that the genome of *H. pylori* strain 51 theoretically encodes for 1,454 ORFs (1). However, the total number of protein spots generated via 2-DE may be more than theoretical ORFs, as a number of proteins can be processed by proteolytic enzymes or modified via methylation, glycosylation, and phosphorylation (18~21). In this study, the sarcosine-insoluble proteins of *H. pylori* strain 51 evidenced more than 20 silver-stained spots in the 2-DE gel using an IPG strip (17 cm) of pH 6.0~11.0 when analyzed using PDQUEST 2-D Gel Analysis Software. The majority of the protein spots were positioned in the alkaline p*I* regions and their molecular weights were between 10~100 kDa. *H. pylori* OMPs were detected within the alkaline p*I* region of the 2-DE gel. This may be reflective of evolutionary pressure for high alkaline proteins, owing to the acidic environment of the organism (16). Among the 20 protein spots processed in this study, some spots did not generate apparent spectra on Q-TOF-MS. The spectra of several protein spots could not be generated on Q-TOF-MS, even though they exhibited intense spots. The remaining 15 spots could be applied to protein identification. From the Q-TOF-MS spectrum of an individual spot, 15~20 peptide peaks, the count intensities of which were 4-fold higher than that of the noise, were utilized to search the database. The molecular weights and p*I* values were estimated in the 2-DE gels and compared with their theoretical masses and p*I* values. The values of the gel-estimated molecular weights closely matched their theoretically predicted values. The gel-estimated p*I* values corresponded strongly with the calculated values of the protein spots. However, there were some slight variations in the protein spots showing p*I* regions with pH values in excess of 6.0. Posttranslational-proteolytic processing and modification may be one possible cause for these discrepancies. Differences between the gel-estimated and theoretical p*I* values were larger than those between the gel-estimated and theoretical molecular weights, thus indicating that processing and modification may affect the p*I* values of proteins (14).

In this study, we identified 15 protein spots corresponding to 9 genes, as shown in Table 1. Among the 30 spots, 4 horizontally located spots (spot no 1, 2, 4, 5) were identified as a KHP0853 homologous to OMP21 in *H. pylori* 26695. KHP0852 and KHP1353 are homologous to OMP20 and OMP32, respectively. KHP1017 was identified as a γ-glutamyltranspeptidase (GGT), an enzyme that catalyzes the transpeptidation reaction in which a γ-glutamyl moiety is transferred from γ-glutamyl compounds, such as glutathione and glutathione-conjugated compounds, to amino acids and a protein of 61 KDa, although the observed value (38 KDa) was smaller than anticipated. Mature GGT is a heterodimer (38 KDa + 20 KDa) and a periplasmic enzyme, suggesting that the spot may represent the large subunit of this periplasmic protein (22). KHP 0172 was identified as cell binding factor 2, located in the OM. Two horizontally located spots (spot no 21, 22) were identified as a hypothetical protein (KHP1069) homologous to HP1173 in *H. pylori* 26695. HP1173 is secreted into the extracellular medium and was identified previously as an immunoreactive protein (23).

Additionally, KHP0614 was identified as a hypothetical

protein. However, the functions of this protein are not currently known. Some horizontally aligned spots were identified. This horizontal separation may be attributable to post-translational modifications that result in differentially-charged side chains on the amino acid residues of one species of protein (24).

In summary, certain proteins, including the putative OMPs, were identified by the enrichment of sarcosine-insoluble proteins from *H. pylori* 51. In this study, the identified proteins appeared to assume a pattern similar to that of *H. pylori* strain 26695, and the 9 genes may be major OMPs of *H. pylori* 51. Our results contribute substantially to the characterization of the OMPs of *H. pylori* 51, which will facilitate the identification of new potential target proteins of the Korean *H. pylori* strain 51 for vaccine development and drug therapy.

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