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Proteomic Analysis of *Helicobacter pylori* Whole Cell Proteins using the Narrow Range IPG Strips

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It has been reported that most of *Helicobacter pylori* proteome components appear so crowded in the region of pH 4.5~8.0 that a lot of them were inseparable in 2-DE using the broad range IPG strip. Therefore, inseparable protein spots in 2-DE profiles have to be apart from each other for improving the protein identification. Here, we attempt to examine the usability of the narrow range IPG strips for separating close spots in the broad range IPG strip at proteomic analysis of *H. pylori*. The whole cell proteins of *H. pylori* strain 26695 were separated by narrow range IPG strips (p*I* 3.9~5.1, 4.7~5.9, 5.5~6.7, and 6.3~8.3, respectively), followed by SDS-PAGE, and visualized by silver staining, showing that the distances between spots were widened and the total number of detectable spots was increased. Resolved protein spots were identified by the peptide fingerprinting using MALDI-TOF-MS. As a result, 87 expressed proteins were identified by the peptide fingerprinting. Of them, 23 proteins, including hydrogenase expression/formation protein, purine-binding chemotaxis protein, and ribosomal protein S6, have not been reported in the previous proteome studies of *H. pylori*. Thus, these results demonstrate that the high complexity proteome components could be effectively separated using the narrow range IPG strips, which might be helpful to strengthen the contents of the master protein map of the *H. pylori* reference strain.

Key Words: Narrow range IPG strip / *Helicobacter pylori* / Proteomics

INTRODUCTION

Helicobacter pylori, a gram-negative spiral bacterium, was first observed in human gastric mucosa in 1982 by Marshall and Warren (22). The discovery of *H. pylori* in the gastric mucosa has provided a new paradigm for understanding the pathogenesis and control of most gastric disorders including peptic ulcer and gastric cancers (5,7,13). Gastric mucosa with *H. pylori* challenges are destined to develop inflammatory responses resulting in accumulating reactive oxygen radicals which damage DNAs of gastric tissues to lead pathological events. Although the severity of gastric diseases caused by *H. pylori* varies from asymptomatic to ulcerative or malignant (3,8), most *H. pylori* infec-

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tions cause only benign subclinical symptoms and do not progress to serious diseases (27,28). Since more than half of the world's population suffers from *H. pylori* infection (3,6,31), gastric diseases caused by *H. pylori* infection might be the most common human disorders.

Genome sequencing of *H. pylori* strains has been initiated for delving into their biochemistry and metabolism, which may provide novel drug targets for the development of monotherapies against *H. pylori*. The entire genome sequences of *H. pylori* strains 26695, J99, HPAG1 have been reported (1,26,32). The accumulation of genomic information, combined with advances in protein separation and identification techniques, has advanced the technology of proteomics (17). High-throughput two-dimensional electrophoresis (2-DE) displays and the identification of the entire protein complement of the genome expressed in an organism can lead to the identification of disease-specific markers or virulent strains of pathogenic bacteria (9). The proteome components of *H. pylori* have been investigated to identify functionally active genes, subcellular proteins, disease-specific proteins, and immunoreactive proteins. The genome of *H. pylori* has been determined to contain about 1,590 open reading frames (ORFs) (1,26,32). Up to date, more than 250 have been identified to list as proteome components of *H. pylori* (4,10,18,20,21,23,29,33), revealing more intensive exploration needed to construct proteomic information of *H. pylori*. Actually, most spots appeared so crowded in the region of p*I* 4.5~8.0 that they were inseparable in 2-DE using the broad range IPG strip. Therefore, a part of spots in the region of p*I* 4.5~8.0 should be separated for proteomic analysis, which has been one of the bottlenecks to construct the master proteome map of *H. pylori*.

Now, the narrow range IPG strips have been commercially available to widen the crowded region of p*I* 4.5~8.0. Here, 4 kinds of narrow range IPG strips covered from p*I* 3.9 to 8.3 were subjected to analyze proteome components of *H. pylori* strain 26695. We found that the narrow range 2-DE of *H. pylori* whole cell protein was useful to separate close spots as well as to detect low abundant proteins resulting in strengthening the contents of the master proteome map of the *H. pylori*.

MATRIALS AND METHODS

1. Bacterial strain and culture conditions

H. pylori strain 26695 was supplied by the *H. pylori* Korean Type Culture Collection (Gyeongsang National University School of Medicine, Jinju, Korea). The frozen *H. pylori* were thawed and grown on brucella agar plate containing 10% bovine serum, vancomycin (10 μg/ml), nalidixic acid (25 μg/ml), and amphotericin B (1 μg/ml) at 37℃ under 10% CO_2 and 100% humidity. After 18 h of culture, the bacterial mass was collected, determining by microscopic observation using Gram staining that most of the cells showed a typical curved shape.

2. Two-dimensional electrophoresis and image analysis

Two-dimensional electrophoresis (2-DE) sample preparation was carried out as described previously (10). Cultured cells were lysed using a buffer containing 9.5 M urea, 4% CHAPS, and 35 mM Tris-HCl (pH 7.2). The solubilized protein samples (300 μg) were mixed with the rehydration solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), to a final volume of 320 μl and applied to narrow range IPG strips (17 cm; pH 3.9~5.1, 4.7~5.9, 5.5~6.7, and 6.3~8.3; Bio-Rad, Hercules, CA, USA) in a re-swelling tray (Bio-Rad). After the IPG strips were rehydrated, IEF was performed using a Protein IEF Cell (Bio-Rad) and three preset programs consisting of the first conditioning step (15 min at 250 V), the linear voltage ramping step (3 h at 10,000 V), and the maximum voltage ramping step of up to 90,000 Vh. The current did not exceed 50 μA per strip. Following IEF, the strips were equilibrated with 0.375 M Tris buffer (pH 8.8) containing 6 M urea, 2% SDS, 20% glycerol, 2% DTT, and 0.01% bromophenol blue. The strips were equilibrated again with the same buffer supplemented with 2.5% iodoacetamide. The second dimension SDS-PAGE was carried out overnight at 20 mA per gel using a 12.5% separating polyacrylamide gel. The resolved protein spots on the gels were visualized by silver staining and scanned using a Fluor-S MultiImager (Bio-Rad).

3. Destaining and in-gel digestion of protein spots

The silver-stained spots were excised from the 2-DE gels and transferred into microcentrifuge tubes. The spots were destained with fresh chemical reducers in a 1:1 ratio of 30 mM potassium ferricyanide and 100 mM sodium

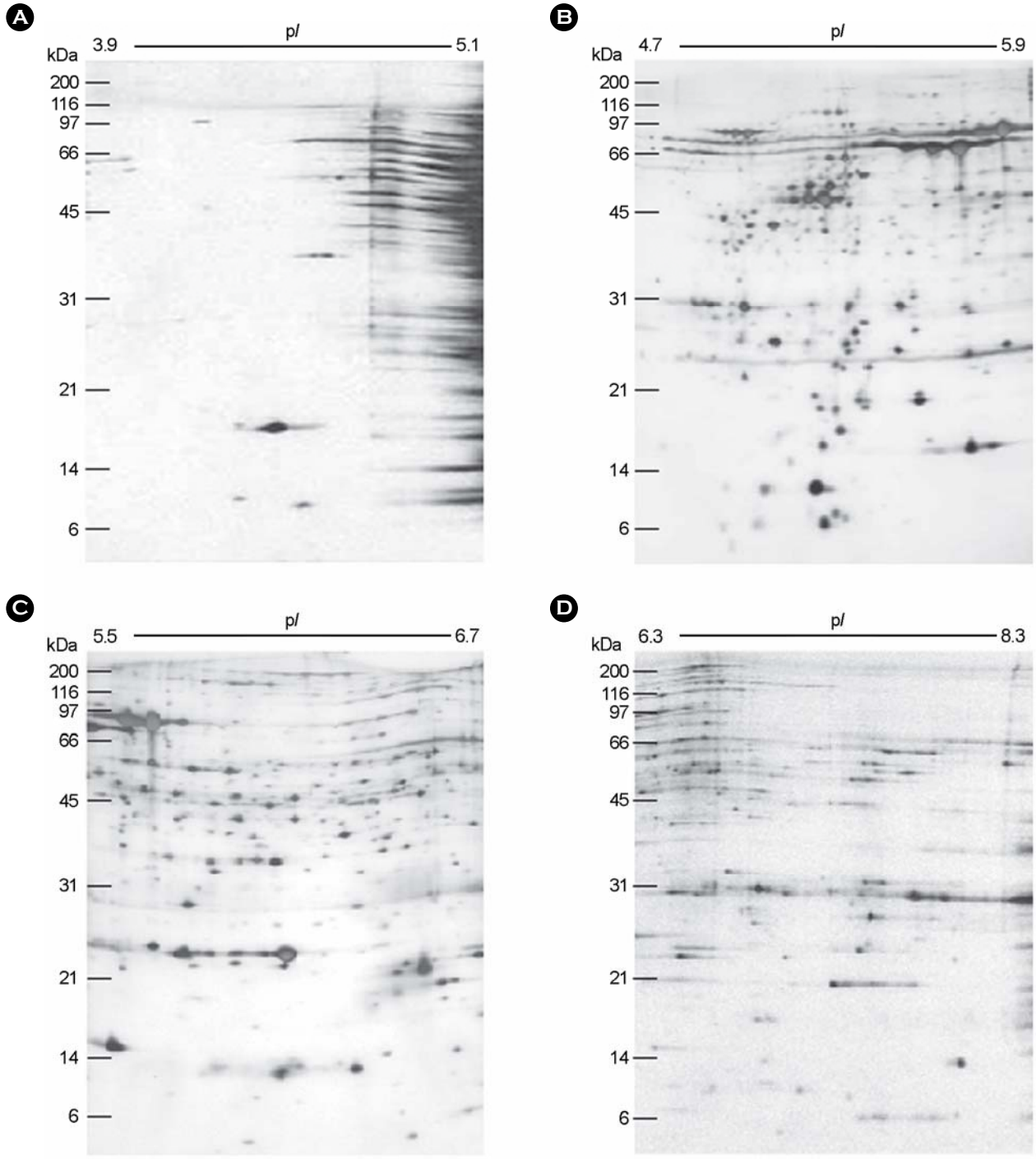

Figure 1. Two-dimensional-gel electrophoresis of whole cell proteins from *H. pylori* strain 26695. Whole cell proteins solution was separated on a narrow range IPG strips, followed by 12.5% SDS-PAGE, and visualized by silver staining. The original gel size was 20×18×0.15 cm. **(A)** pH 3.9~5.1; **(B)** pH 4.7~5.9; **(C)** pH 5.5~6.7; (**D)** pH 6.3~8.3.

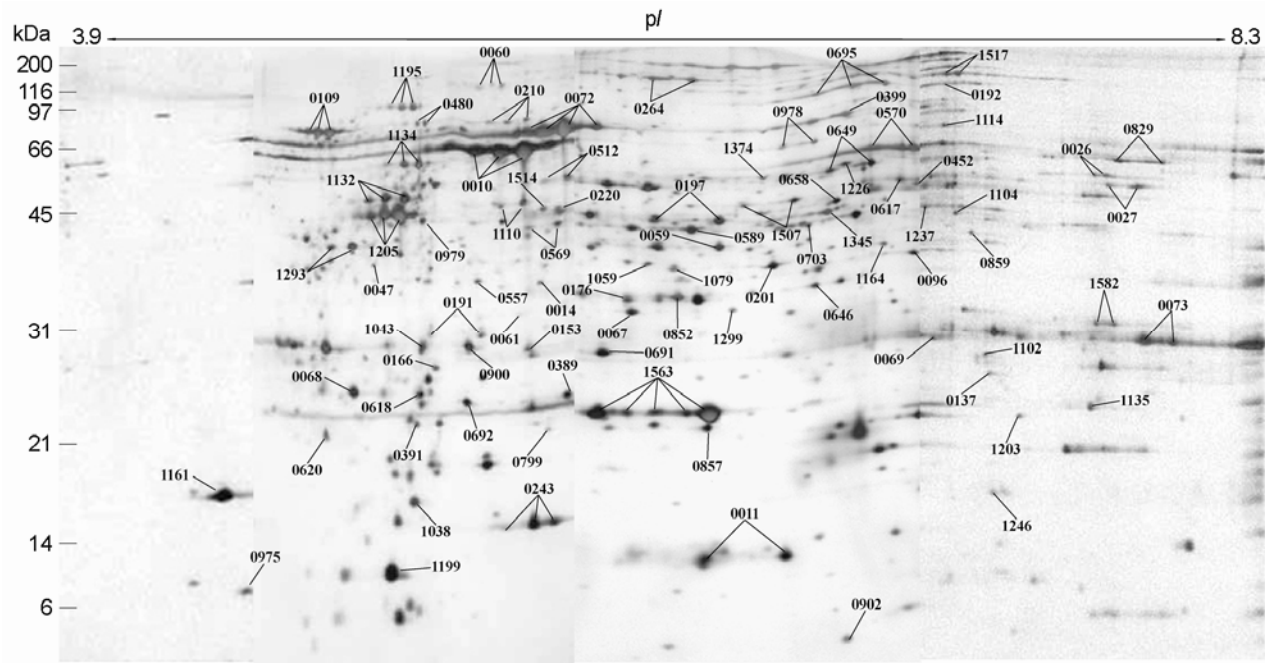

Figure 2. The composite profile of the whole cell proteins of *H. pylori* strain 26695 resolved in the 2-DE using the narrow pH range IPG strips. The whole cell proteins of *H. pylori* strain 26695 was subjected to 2-DE using IPG strips of 3.9~5.1, 4.7~5.9, 5.5~6.7, and 6.3~8.3 ranges, the gels were visualized with silver staining. The 4 kinds of images were taken up and analyzed with PDQUEST software, and excluded with the overlapping zones of each right and left termini, and then combined by PHOTOSHOP software to generate the composite one ranged from p*I* 3.9 to p*I* 8.3. The marked protein spots have been identified and listed in Table 1.

thiosulfate, as described previously (10), with occasional mixing until the brownish color disappeared. The gel pieces were rinsed three times with distilled water to stop the reaction. Ammonium bicarbonate (500 μl of 200 mM) was added to cover the gels for 20 min and then discarded. The gel pieces were dehydrated with 100 μl of acetonitrile and dried in a vacuum centrifuge. An in-gel digestion was carried out by the method described by O'Connell and Stults (25). Gel pieces were rehydrated by covering with digestion buffer containing trypsin (12.5 ng/ml) and incubated on ice for 45 min. The enzyme solution was replaced with 20 μl of the buffer without enzyme and incubated overnight at 37℃. The digested solutions (20 μl) were transferred into clean E-tubes and dried in a vacuum. The resulting pellets were dissolved in 2 μl of 0.1% trifluoroacetic acid (TFA).

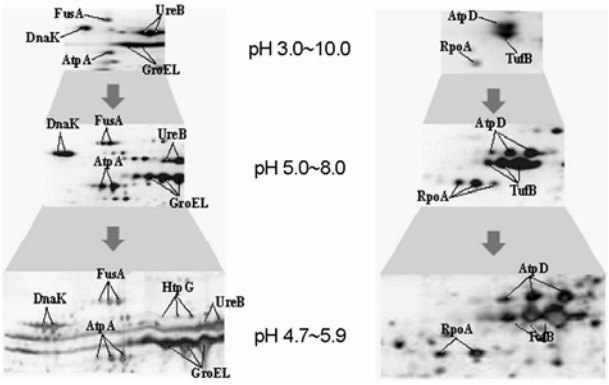

Figure 3. Blow-up images of two different regions in 2-DE gels of *H. pylori* whole cell proteins resolved by broad (pH 3.0~10.0), intermediate (pH 5.0~8.0), narrow (pH 4.7~5.9) pH strips. Here, the narrower is the pH range of the strip, the higher resolution does the spots profile show in which the resolution and the number of spots are improved. HtpG spot appears in the gel of narrow pH strips but did not in otherwise.

4. Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS)

For the matrix solution, α-cyano-4-hydroxycinnamic acid (40 mg/ml) was dissolved in 50% acetonitrile and 0.1% TFA. The matrix and sample solutions (2 μl each) were mixed and loaded into the target wells, rapidly dried, and washed using deionized water. The wells were dried for 10 min at room temperature and subjected to MALDITOF-

Table 1. Protein identification of spots from narrow range 2-DE gels the whole cell proteins of *H. pylori* strain 26695

TIGR locus[§]	MW (Da)	p*I*	Annotation
HP 0010	58263.91	5.4772	Chaperone and heat shock protein (groEL)
HP 0011	12990.87	6.5917	Co-chaperone (groES)
HP 0014	31681.02	5.7841	Hypothetical protein
HP 0026	48350.02	8.0209	Citrate synthase (gltA)
HP 0027	47531.05	7.9527	Isocitrate dehydrogenase (icd)
HP 0047*	36405.28	4.8329	Hydrogenase expression/formation protein (hypE)
HP 0059*	32723.51	4.8431	Hypothetical protein
HP 0060*	93128.96	4.8450	Hypothetical protein
HP 0061*	21764.88	5.9064	Hypothetical protein
HP 0067	29722.06	6.1629	Urease accessory protein (ureH)
HP 0068	21955.25	4.7602	Urease accessory protein (ureG)
HP 0069	28618.77	7.6498	Urease accessory protein (ureF)
HP 0072	61683.52	5.9110	Urease beta subunit (ureB)
HP 0073	26539.49	8.9593	Urease, alpha subunit (ureA)
HP 0096	34796.18	7.0468	Phosphoglycerate dehydrogenase
HP 0109	67051.91	4.7568	Chaperone and heat shock protein70 (dnaK)
HP 0137*	23610.48	7.6666	Hypothetical protein
HP 0153*	37686.28	5.5065	Recombinase (recA)
HP 0166	25855.46	5.0781	Response regulator (ompR)
HP 0176	33772.69	6.2517	Fructose-bisphosphate aldolase (tsr)
HP 0191	27651.76	5.1972	Fumarate reductase, iron-sulfur subunit (frdB)
HP 0192	80121.10	7.3217	Fumarate reductase, flavoprotein subunit (frdA)
HP 0197	42362.06	6.4292	S-adenosylmethionine synthetase 2 (metX)
HP 0201	36483.11	6.5193	Fatty acid/phospholipid synthesis protein (plsX)
HP 0210	71274.03	5.2608	Chaperone and heat shock protein C62.5 (htpG)
HP 0220	42403.38	6.1542	Synthesis of [Fe-S] cluster (nifS)
HP 0243	16933.34	5.8309	Neutrophil activating protein (napA) (bacterioferritin)
HP 0264	96683.36	6.2660	ATP-dependent protease binding subunit (clpB)
HP 0389	24617.63	6.1683	Superoxide dismutase (sodB)
HP 0391*	18966.68	5.0808	Purine-binding chemotaxis protein
HP 0399	62826.43	6.7727	Ribosomal protein S1 (rps1)
HP 0452*	60506.79	5.9621	Conserved hypothetical protein
HP 0480	66676.31	5.0937	GTP-binding protein, fusA-homolog (yihK)
HP 0512	54513.58	6.0379	Glutamine synthetase (glnA)
HP 0557	34881.13	5.3110	Acetyl-coenzyme A carboxylase (accA)
HP 0569	40574.45	5.5302	GTP-binding protein (gtp1)
HP 0570	54433.07	7.1054	Aminopeptidase a/i (pepA)
HP 0589	41508.81	6.3623	Ferredoxin oxidoreductase, alpha subunit
HP 0617	65601.53	6.8834	Aspartyl-tRNA synthetase (aspS)

Table 1. Continued

TIGR locus[§]	MW (Da)	p*I*	Annotation
HP 0618	21243.21	5.0039	Adenylate kinase (adk)
HP 0620	19271.89	4.8028	Inorganic pyrophosphatase (ppa)
HP 0646*	30975.69	6.5192	UDP-glucose pyrophosphorylase (galU)
HP 0649	51980.86	6.8341	Aspartate ammonia-lyase (aspA)
HP 0658	53288.14	6.0094	Glutamyl-tRNA(Gln) amidotransferase subunit B (gatB)
HP 0691	25362.24	5.8082	3-oxoadipate coA-transferase subunit A (yxjD)
HP 0692	22262.85	5.3450	3-oxoadipate coA-transferase subunit B (yxjE)
HP 0695	78532.79	6.9435	Hydantoin utilization protein A (hyuA)
HP 0703	43414.34	6.7535	Response regulator
HP 0799*	19676.86	5.3363	Molybdopterin biosynthesis protein (mog)
HP 0829	51801.80	8.2155	Inosine-5'-monophosphate dehydrogenase (guaB)
HP 0852*	35776.74	6.6806	Hypothetical protein
HP 0857*	21102.26	6.3967	Phosphoheptose isomerase (gmhA)
HP 0859	37412.66	7.3588	ADP-L-glycero-D-mannoheptose-6-epimerase (rfaD)
HP 0900	27310.33	5.2449	Hydrogenase expression/formation protein (hypB)
HP 0902	11029.96	6.6745	Hypothetical protein
HP 0975*	10656.15	4.7389	Glu-tRNA(Gln) amidotransferase, subunit C
HP 0978	54492.59	6.6324	Cell division protein (ftsA) protein
HP 0979*	40954.34	5.1659	Cell division protein (ftsZ)
HP 1038	18483.25	4.9294	3-dehydroquinase type II (aroQ)
HP 1043*	25468.16	4.9740	Response regulator
HP 1059	37360.31	6.0818	Holliday junction DNA helicase (ruvB)
HP 1079*	42919.70	6.3237	Hypothetical protein
HP 1102	25727.47	7.3447	Glucose-6-phosphate 1-dehydrogenase (devB)
HP 1104	38645.51	7.4067	Cinnamyl-alcohol dehydrogenase ELI3-2 (cad)
HP 1110	44743.62	5.8122	Pyruvate ferredoxin oxidoreductase, alpha subunit
HP 1114	75916.20	6.9096	Excinuclease ABC subunit B (uvrB)
HP 1132	51478.08	5.0767	ATP synthase F1, subunit beta (atpD)
HP 1134	55143.11	5.0512	ATP synthase F1, subunit alpha (atpA)
HP 1135*	20368.81	8.4730	ATP synthase F1, subunit delta (atpH)
HP 1161	17492.37	4.2040	Flavodoxin (fldA)
HP 1164	35985.98	6.8739	Thioredoxin reductase (trxB)
HP 1195	77020.88	5.0111	Translation elongation factor EF-G (fusA)
HP 1199	13313.38	4.9125	Ribosomal protein L7/L12 (rpl7/l12)
HP 1203	20261.24	7.8735	Transcription termination factor NusG (nusG)
HP 1205	43647.70	4.9302	Translation elongation factor EF-Tu (tufB)
HP 1226*	40196.14	6.5108	Oxygen-independent coproporphyrinogen III oxidase (hemN)
HP 1237*	41635.41	7.1579	Carbamoyl-phosphate synthetase (pyrAa)
HP 1246*	16971.34	7.4983	Ribosomal protein S6 (rps6)

Table 1. Continued

TIGR locus§	MW (Da)	p*I*	Annotation
HP 1293	38499.11	4.6887	DNA-directed RNA polymerase, alpha subunit (rpoA)
HP 1299	27576.93	6.4208	Methionine amino peptidase (map)
HP 1345*	44771.64	6.7477	Phosphoglycerate kinase
HP 1374*	50353.20	6.4377	ATP-dependent protease ATPase subunit (clpX)
HP 1507*	43904.36	6.5698	Conserved hypothetical ATP-binding protein
HP 1514	44649.63	5.5915	Transcription termination factor NusA (nusA)
HP 1517	149715.58	7.5020	Type IIS restriction enzyme R and M protein (ECO57IR)
HP 1563	22235.57	6.2482	Alkyl hydroperoxide reductase (tsaA)
HP 1582	29720.44	8.0532	Pyridoxal phosphate biosynthetic protein J (pdxJ)

§ Tigr loci from figure 2., * Not previously identified in references

MS analysis using a Voyager Biospectrometry Workstation (PE Biosystems, Foster City, CA, USA) with the following parameters: 20 kV accelerating voltage, 75% grid voltage, 0.02% guide wire voltage, 70 ns delay, and a mass gate from 800 to 3500. The peptide mass fingerprints were analyzed using the program MS-FIT of ProteinProspect developed by the UCSF Mass Spectrometry Faculty (http://prospector.ucsf.edu). The NCBI database of *Helicobacter* proteins was searched to identify the proteins, using monoisotopic peptide masses and allowing a molecular mass range of 2DE±15%, a peptidemass accuracy of 50 ppm, and one partial cleavage. When matching proteins were not found, the molecular mass window was extended. Pyroglutamic acid modification of *N*-terminal glutamine, oxidation of methionine, and acrylamide modification of cysteine were taken into consideration.

RESULTS

1. Two-DE profiles of *H. pylori* proteome using narrow range IPG strips

Previous papers have described 2-DE proteome maps of *H. pylori* strain 26695 in which most abundant protein spots were clustered in the p*I* regions of pH 4.5~8.0 (10, 18). Here, the narrow range IPG strips were subjected to widen closed spots in order to get highly resolved profiles in 2-DE of whole cell proteins of *H. pylori* strain 26695. The whole cell proteins solution of the strain was loaded onto precast IPG strips (17 cm) of the narrow pH range for the first-dimensional separation. The strips were loaded onto a 12.5% acrylamide gel of 18×20 cm for the second-dimensional electrophoresis. After running SDS-PAGE, the separated spots were visualized by silver staining, as shown in Fig. 1. Each of 2-DE gels was excluded by the overlapping regions and combined together to the composite gel (Fig. 2). Fig. 3 represented actual examples in which the narrower had the pH range of the strip, the higher resolution did the spots profile show. Therefore, the narrow range IPG strips made close spots become further apart from each other as well as weak spots hidden by strong spots appear to be independent spots.

2. Peptide mass fingerprinting and protein identification

As shown in Fig. 2, the silver-stained spots generated by 2-DE using the narrow range IPG strips were numbered, excised, destained, and followed by in-gel digestion using trypsin for peptide fingerprinting. The mass of the resulting peptide mixtures was measured by MALDI-TOF-MS. Among the all represented proteins spots processed, proteins expressed by 87 genes were identified as shown in Table 1. Of them, 64 proteins like urease beta subunit, 60 kDa chaperonin, and alkyl hydroperoxide reductase had been identified in the previous studies (4,10,18,20,21,23,29,33). And 23 proteins that have not been reported in the previous proteome studies of *H. pylori* could be identified like hydro-

genase expression/formation protein, purine-binding chemotaxis protein, and ribosomal protein S6.

DISCUSSION

The 2-DE method is capable of simultaneously separating thousands of proteins in cells, tissues, or whole organisms (15,16). However, technical limitation has always been met since a lot of gene products have too similar physicochemical properties to separate each other in 2-DE experiment. Several researches for exploiting proteome components of *H. pylori* demonstrated that most of spots were closely displayed together in the region of pH 4~8 and only part of them could be identified by protein fingerprinting. Protein identification of a given spot might be hampered by contamination of nearby spots. Therefore, physical separation of close spots is important to identify protein spots successfully. Broad range 2-DE gels, which has generally used in proteomic researches of *H. pylori*, have been proved to be insufficient to construct a proteome map of *H. pylori*. Now, additional approaches were needed for enhancing detection of low abundance proteins and increasing the represented protein spots on the gel (30,35). Here, the narrow range pH IPG strips were applied for improving the resolution of spots profiles displayed in 2-DE. The narrow range pH IPG strips will take the increase of the number of protein spots per pH unit for grant (34). Also, 2-DE using the narrow range IPG strips was reducing overlap of close spots resulting in improving the purities of isolated spots to the quality enough for protein identification.

For the understanding of biomarkers involved in pathology, it is necessary to identify low abundant components of the proteome. Previous reports have described that the whole cell proteins of *H. pylori* strain 26695 has been resolved to show 1,000~1,800 spots in 2-DE and MALDI-TOF-MS peptide-fingerprinting analysis has identified about 250 genes products (4,10,18,20,21,23,29,33). However, many protein spots remained to be identified in p*I* 4.5~8.0 region in order to construct a master proteome map of *H. pylori*. In this study, high complexity region (p*I* 4.5~8.0) was separated by using the narrow IPG strips (Fig. 1). As result, the distances between spots were widened and total number of detectable spots was increased when compared with previous investigations. And with removing the overlap regions of right and left termini, images of narrow range IPG gels were combined to the composite gel by using PDQUEST and PHOTOSHOP software (Fig. 2).

Comparison of images obtained from 3~10, 5~8, and 4.7~5.9 pH strips demonstrated that the narrow range IPG strips made the distance among nearby spots widen and spots hidden by the large spots appear (Fig. 3). These results will be helpful to strengthen the purity of picked spots to the quality enough for protein identification and to find low abundant spots. Proteins expressed by 87 genes could be identified from spots displayed in the narrow range pH IPG strips, of which 23 proteins had not been reported by other investigations (Fig. 2, Table 1) (4,10,18,20,21,23,29,33). Eight of newly identified proteins were of unknown function and 6 were associated with cellular processes and signaling, 6 with metabolism, and 3 with information storage and processing. Especially, RecA protein (HP0153) was known to be the central components of homologous recombination machinery and of the SOS system in most bacteria. The RecA protein of *H. pylori* has likewise been shown to be necessary for DNA repair (12). UDP-glucose pyrophosphorylase (HP0646) catalyzes the synthesis of UDP-glucose. UDP-glucose was known to be an essential metabolite in almost all cellular processes in living organisms and to be an essential protein for virulence in various Gram-negative bacteria (13).

In conclusion, the whole cell proteins of *H. pylori* strain 26695 were displayed using the narrow IPG strips and then identified by peptide fingerprints. This investigation showed that the narrow range IPG strips might contribute to strengthen the contents of master proteome map of the *H. pylori* reference strain.

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