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Comparative proteome analysis of *Helicobacter pylori* clinical strains by two-dimensional gel electrophoresis*

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Abstract: Objective: To investigate the pathogenic properties of *Helicobacter pylori* by comparing the proteome map of *H. pylori* clinical strains. Methods: Two wild-type *H. pylori* strains, YN8 (isolated from biopsy tissue of a gastric cancer patient) and YN14 (isolated from biopsy tissue of a gastritis and duodenal ulcer patient), were used. Proteomic analysis, using a pH range of 3–10 and 5–8, was performed. The individual proteins were identified by quadrupole time-of-flight (Q-TOF) mass spectrometer and protein database search. Results: Variation in spot patterns directed towards differential protein expression levels was observed between the strains. The gel revealed prominent proteins with several protein "families". The comparison of protein expressions of the two strains reveals a high variability. Differentially present or absent spots were observed. Nine differentially expressed protein spots identified by Q-TOF included adenosine triphosphate (ATP)-binding protein, disulfide oxidoreductase B (DsbB)-like protein, N utilization substance A (NusA), ATP-dependent protease binding subunit/heat shock protein, hydantoin utilization protein A, seryl-tRNA synthetase, molybdenum ABC transporter ModD, and hypothetical proteins. Conclusions: This study suggests that *H. pylori* strains express/repress protein variation, not only in terms of the virulence proteins, but also in terms of physiological proteins, when they infect a human host. The difference of protein expression levels between *H. pylori* strains isolated from gastric cancer and gastritis may be the initiator of inflammation, and result in the different clinical presentation. In this preliminary study, we report seven differential proteins between strains, with molecule weights from approximately 10 kDa to approximately 40 kDa. Further studies are needed to investigate those proteins and their function associated with *H. pylori* colonization and adaptation to host environment stress.

Key words: *Helicobacter pylori*, Proteome, Gastric cancer, Gastritis, Two-dimensional gel electrophoresis
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1 Introduction

Gastric cancer is the fourth most common cancer and the second most common cause of cancer deaths (Parkin *et al*., 2005). There is a multistep progression stage from pre-malignancy to invasive malignancy. Thus, early gastric cancer diagnosis for effective preventive strategies and therapeutics against gastric cancer is urgently required. Many epidemiological studies have shown a positive relationship between *Helicobacter pylori* infection and the risk for gastric cancer. Experimental studies using Mongolian gerbils showed that *H. pylori* infection increases the risk for gastric cancer (Kikuchi, 2002). Based on results of epidemiological and clinical studies, the World Health Organization (WHO) has declared *H. pylori* as a definitive carcinogen in 1994.

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The bacterium *H. pylori* was first isolated from a patient with active chronic gastritis in 1983 (Marshall and Warren, 1984). It is now clear that *H. pylori* inflammation is associated with stomach disease, such as gastritis, gastric and duodenal ulcer, and gastric cancer. The detection of *H. pylori* in clinical practice is generally by either an invasive method, requiring endoscopy (Hp culture, histology, and urease test of biopsy) or via non-invasive methods ($^{13}C/^{14}C$-urea breath test, Hp antibody serological test, PCR-Hp-DNA test, and Hp stool antigen (HpSA)).

The mechanism by which *H. pylori* infection induces carcinogenesis is still unclear. The evidence comes mainly from epidemiological studies supporting that the risk for development of stomach diseases is higher in persons infected with *cag* pathogenicity island (*cag*PAI)-positive *H. pylori* than in those infected with *cag*PAI-negative strains (Blaser *et al*., 1995; van Doorn *et al*., 1998; Blaser and Berg, 2001). After cytotoxin-associated antigen (CagA) encoded by the *cag*PAI is translocated into gastric epithelial cells, it causes cellular alterations, including cell morphological changes, dephosphorylation of CagA protein, and increased levels of interleukin-8 (IL-8) (Zhang *et al*., 2009). Gastric epithelial cell damage may be a consequence of the inflammatory responses induced by *H. pylori* infection. *cagA* and *iceA* have been proposed as biomarkers that might predict the risk for symptomatic clinical outcomes. However, they cannot explain why *H. pylori* strains isolated from asymptomatic patients have also the same frequency of expression of both CagA and VacA compared to the strains isolated from patients with peptic ulcer or gastric cancer (Park *et al*., 1998; Yamaoka *et al*., 1999). Neither *iceA* nor assembly of *iceA*/*vacA*/*cagA* is helpful in predicting the clinical presentation infected with *H. pylori*. *H. pylori* strain-specific factors may influence the pathogenicity of different *H. pylori* isolates and the presentation of a clinical outcome. There should be other proteins/factors cooperating with CagA and VacA to induce or promote the development of disease.

The complete genome of strains (Tomb *et al*., 1997; Alm *et al*., 1999) provides sufficient genetic information for proteome analysis of *H. pylori*. Proteome technology employs protein separation by two-dimensional gel electrophoresis (2-DE) followed by identification using mass spectrometry (MS) methods (Jungblut *et al*., 2000; 2008; Graves and Haystead, 2002; Govorun *et al*., 2003; Friedman *et al*., 2009; Schmidt *et al*., 2009). The fact that 35 genes of *H. pylori* 11637 translate 93 proteins (Lock *et al*., 2001) suggests that *H. pylori* proteins express a high degree of post-translational modification. A comparative proteome map of *H. pylori* strains should be beneficial to investigate the pathogenic properties of these organisms. In this preliminary study, by comparing 2-DE proteome maps of *H. pylori* isolates obtained from gastric cancer and gastritis, we evaluate the variability of the protein expression level between the two strains.

2 Materials and methods

2.1 *H. pylori* strains

Two wild-type *H. pylori* strains, YN8 (isolated from biopsy tissue of a gastric cancer patient) and YN14 (isolated from biopsy tissue of a gastritis and duodenal ulcer patient), were used in this study. The isolates were confirmed by microscopy, culture, and rapid urease test (Sanqiang Biochemistry, Fujian, China).

2.2 *H. pylori* and protein preparations

H. pylori strains were grown on Columbia agar base (Oxoid Ltd.) including amphotericin B (2.5 μg/ml), trimethoprim (2.5 μg/ml), and vancomycin (5 μg/ml) supplemented with 10% sheep blood at 37 °C in a microaerobic condition (GENbox, bioMérieux). Then *H. pylori* clones were cultivated in Brucella broth (Becton Dicknson) containing 10% horse serum. Bacteria were washed in phosphate-buffered saline (PBS) (Govorun *et al*., 2003). The pellet of bacteria was lysed with lysis buffer (0.04 g/ml 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 8 mol/L urea, 65 mmol/L dichlorodiphenyltrichloroethane (DDT), 2% (v/v) Bio-Lyte, proteinase inhibitor) for 15 min. The suspension was sonicated (Microson™ Ultrasonic Cell Disruptor) for 20 s and followed by centrifugation at 1500 r/min for 60 min. The supernatants were removed and stored at −80 °C.

2.3 Protein assay

Protein concentrations were detected by a protein

assay kit (Bio-Rad). In brief, 10 μl of sample and bovine serum albumin (BSA) control sample (0.4 mg/ml) were added to a 96-cell microplate. Then 200 μl of diluted dye reagent was added. The microplate was mixed and incubated at room temperature for at least 5 min. Protein concentrations were calculated by measurement of absorbance at 595 nm ($CERES_{900}$ Bio-Tek Instruments Inc.) with BSA as a standard.

2.4 Two-dimensional gel electrophoresis

First-dimensional runs were performed using nonlinear immobilized pH gradient (IPG) strips (Bio-Rad). Protein was loaded into isoelectric focusing (IEF) gel (pI 5–8) (Bio-Rad). A total of 100 μg of protein and 1 mg of protein were used for silver staining and Coomassie brilliant blue staining, respectively. Protein equilibrium and isoelectrofocusing were carried as described previously (Govorun *et al*., 2003). For second-dimensional runs, 10% gel was used according to molecular weight. The proteins were detected by silver staining for analysis and by Coomassie brilliant blue staining for identification. The PDQuest software (Bio-Rad) was used for protein spot matching analysis.

2.5 In-gel digestion

According to recommended protocols (Bio-Rad), gel fragments (1 mm^3) were cut out of the protein spots stained with Coomassie using a sterilized knife. Each fragment was washed twice in 200 μl of 50% (v/v) acetonitrile (CH_3CN), then washed in 100 mmol/L NH_4HCO_3 containing 30% CH_3CN for 45 min, 50 mmol/L NH_4HCO_3 containing 50% CH_3CN for 30 min, and 25 mmol/L NH_4HCO_3 containing 50% CH_3CN for 20 min. Each dried gel fragment was treated with 10 μl of trypsin (20 ng/μl) (Promega, USA), and then incubated in 20 μl of NH_4HCO_3 (25 mmol/L) at 37 °C for 20 h. After the trypsinolysis, gel fragments were incubated in 50 μl of H_2O for 10 min. Supernatant was collected in separate tubes with 50 μl of 5% (v/v) trifluoroacetic acid (TFA) in 60% (v/v) CH_3CN for 15 min. Samples, concentrated to a final volume 10 μl with 5% TFA in 60% CH_3CN, were used for mass spectrometry analysis.

2.6 Quadrupole time-of-flight (Q-TOF) mass spectrometry analysis and database search

The peptide analysis was carried out as per protocols of the supplier (Bio-Rad). Mass spectra were obtained using the Q-TOF mass spectrometer (Q-TOF Ultima Global mass spectrometer; Waters USA). MS/MS data were searched against NCBInr protein sequence databases (http://www.ncbi.nlm.nih.gov) and Mascot (http://www.matrixscience.com).

3 Results

3.1 Protein expression of *H. pylori* strain

The protein compositions of *H. pylori* YN8 and YN14 were initially separated on 2-DE gel stained by silver staining (Fig. 1). The protein spots were

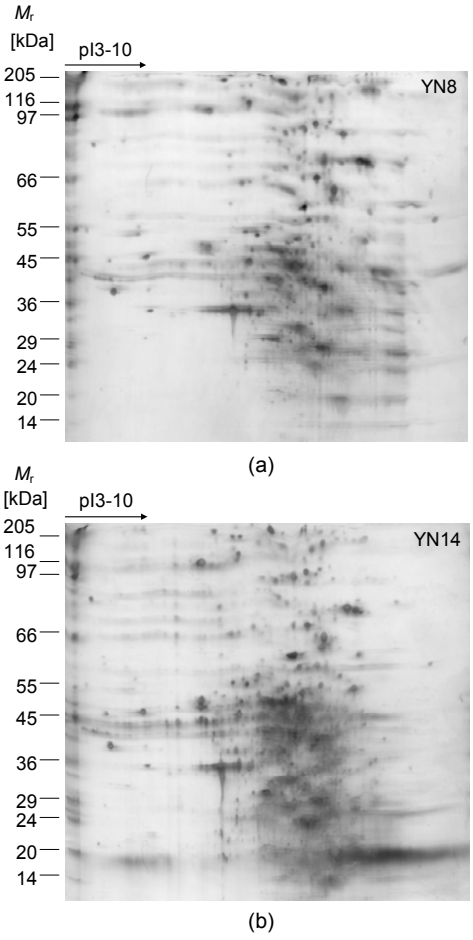

Fig. 1 Proteome maps of *H. pylori* clinical isolates YN8 (a) and YN14 (b)

A total of 100 μg of protein of YN8 or YN14 was applied on 170-mm IPG strips and separated on 10% gels. The 2-DE was stained with silver. The pI 3–10 scale and the molecular weight M_r (in kDa) are indicated on the up and to the left of each panel, respectively

separated over the molecular weight (M_r) range of 10–200 kDa and the pI range of 3–10. The gel revealed prominent individual proteins with several protein "families" (most notably as clusters of bands). Although several main spots/clusters were found at the same position, some proteins visually varied in expression level. Spots around the same pI and M_r were present/absent or strong/weak on the proteome maps of *H. pylori* YN8 and YN14.

3.2 Protein identification

Because most expressed proteins were spread over the center of pI 3–10 gel, we further selected the 2-DE experiments of pI 5–8. The protein comparison of the two strains showed a high variability. High-intensity spots per gel were distinguishable. The differentially expressed protein spots by comparing the two strain proteome maps were excised from the gel stained by Coomassie brilliant blue (circled spots in Fig. 2). Then Q-TOF was performed for protein identification with statistical confidence (Fig. 3). Spot Nos. 1 and 2 showed heavier intensity in YN8 than in YN14. Spot Nos. 3, 4, and 5 were present in YN8, but absent in YN14. Spot Nos. 6, 7, 8, and 9 were shown in YN14, but not in YN8. Seven of nine protein spots identified using protein database (http://www.ncbi.nlm.nih.gov) are adenosine triphosphate (ATP)-binding protein, disulfide oxidoreductase B (DsbB)-like protein, N utilization substance A (NusA), ATP-dependent protease binding subunit/heat shock protein, hydantoin utilization protein A, seryl-tRNA synthetase, molybdenum ABC transporter ModD, and hypothetical proteins (Table 1). Interestingly, the same amino acid sequence has different protein definition depending on individual strain, e.g., IVESDAITALIQR definition is hydantoin utilization protein A in *H. pylori* HPKX, and hypothetical protein in *H. pylori* B128. Two of nine proteins are unknown.

Table 1 Protein identified on the 2-DE analysis of clinical isolates of *H. pylori*

Spot No.*	Clinical isolates		Amino acid sequence	Protein	*H. pylori* strain
	YN8	YN14			
1	↑	↓	MFQQEVTITAPNGLHTRP AAQFVK	Putative secretion/efflux ABC transporter, ATP-binding protein	*H. pylori* Shi470, *H. pylori* J99
				Hypothetical protein	*H. pylori* B128
2	↑	↓	FQQTENQGLNR	Hypothetical protein	*H. pylori* 98-10
				DsbB-like protein	*H. pylori* P12
3	+	−	ITLALAGFR	DsbB-like protein	*H. pylori* P12
				Hypothetical protein	*H. pylori* 98-10
				Transcription termination factor	*H. pylori* G27
				Transcription elongation factor NusA	*H. pylori* HPKX
4	+	−	AQFEEER	Hypothetical protein	*H. pylori* B128
				ATP-dependent protease binding subunit	*H. pylori* P12
				ATP-dependent protease binding subunit/ heat shock protein	*H. pylori* HPKX, *H. pylori* G27
5	+	−	IVESDAITALIQR	Hydantoin utilization protein A	*H. pylori* HPKX
				Hypothetical protein	*H. pylori* B128
6	−	+	DAGFEVIYTGLR	Hypothetical protein	*H. pylori* B128
				Seryl-tRNA synthetase	*H. pylori* G27
7	−	+	YGAGGASIAFLHPK	Hypothetical protein	*H. pylori* 98
				Molybdenum ABC transporter ModD	*H. pylori* P12, *H. pylori* HPKX
8	−	+		Unknown	
9	−	+		Unknown	

Spot Nos. are shown in Fig. 2. Symbols "↑" or "↓" show increased or reduced protein expression intensity, respectively; Symbols "+" or "−" show the presence or absence of a corresponding protein spot on the 2-DE map, respectively

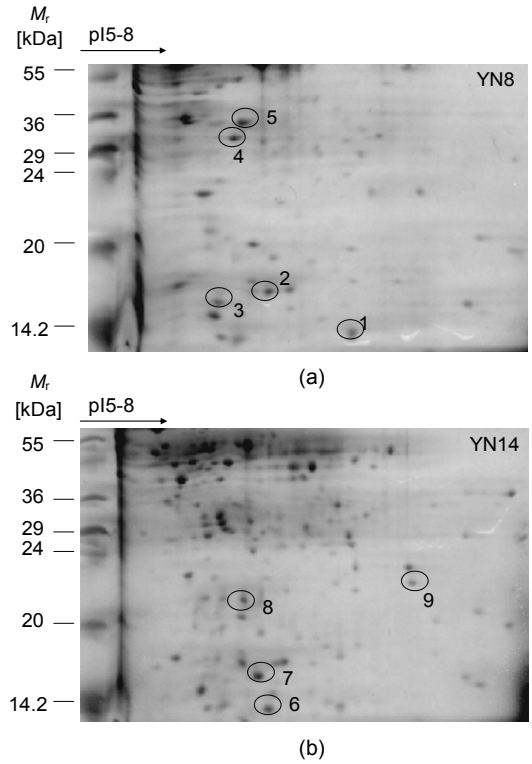

Fig. 2 Proteome maps of *H. pylori* clinical isolates YN8 (a) and YN14 (b)

A total of 1 mg of protein of YN8 or YN14 was applied on 70-mm IPG strips and separated on 10% gels. The 2-DE was stained with Coomassie brilliant blue. The pI 5–8 scale and the molecular weight M_r (in kDa) are indicated on the up and to the left of each panel, respectively. (a) Circled spots (1–5) are proteins that present or strong in intensity compared to YN14; (b) Circled spots (6–9) are proteins that present or strong in intensity compared to YN8

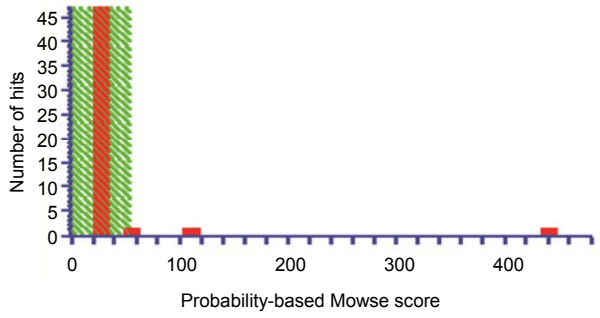

Fig. 3 Identification of protein of Spot 5 by mass spectrometry

Probability-based Mowse score of Spot 5 is shown. Ions score is $-10\times\log P$, where P is the probability that the observed match is a random event. Individual ion scores >54 indicate identity or extensive homology ($p<0.05$). Protein scores are derived from ion scores as a non-probabilistic basis for ranking protein hits

4 Discussion

It is well known that *H. pylori* strains display a high interstrain genomic divergence (Salama *et al*., 2000). This high variation at the genomic level does not provide evidence of a functional protein difference between strains because silent mutations happen naturally. Disordered proteins are considered as disorder-promoting and disease-initiating factors. Thus, we focused on protein expression levels of *H. pylori* isolates.

From this study, different *H. pylori* isolates have individual protein expression levels. The presence or absence of some protein spots on 2-DE was thought to be useful for *H. pylori* infection characterization. The strain-specific proteins act synergistically with virulence factors to reside for decades in the niche of the human stomach, to modulate adaptations to diverse hosts, to cause bacterial persistent infection in hosts, and, finally, to induce different disease outcomes. We speculated that disease-specific proteins were thought to be responsible for the clinical presentation induced by *H. pylori* infection. However, none of the seven identified proteins showed similarities with virulence factors reported in previous studies (Govorun *et al*., 2003; Zhang *et al*., 2009). For example, (1) it has been established that Dsb family (DsbA, DsbB, DsbC, DsbD, and DsbG) of redox proteins is involved in correct formation of disulfide bond into proteins in the periplasm or assembly of many pathogenic virulence factors (Raczko *et al*., 2005). Many bacteria use an oxidative protein-folding machinery to assemble proteins that are necessary for cell integrity and to produce virulence factors. Because *dsb* gene mutations change disulfide bond formation and stability of some extracytoplasmic proteins, Dsb protein is not stable and results in attenuation of pathogens. The interesting thing is that *H. pylori* isolated from gastric cancer showed present or high increased DsbB-like protein compared to that of the strain isolated from gastritis. This infers that a strain which produces much more redox proteins when it colonizes human gastric mucosa for life may portend a higher risk for gastric cancer. This hypothesis needs further study. (2) NusA modulates transcriptional pausing, termination, and antitermination (Paliy *et al*., 2008; Prasch *et al*., 2009; Cohen *et al*., 2009; 2010). The regulatory complexity

of NusA may depend on both the N-terminal domain of NusA and other NusA domains (Ha *et al*., 2010). NusA is also a crucial participant in the phenomenon of stress-induced mutagenesis (Cohen and Walker, 2010). This suggests that bacterium can modulate itself gene transcription in response to stress in a host. (3) Aminoacyl-tRNA synthetases (aaRSs) are enzymes that catalyze the correct attachment of amino acids to their cognate tRNAs in protein synthesis (Lenhard *et al*., 1999; Tumbula *et al*., 1999). Seryl-tRNA synthetase in *Escherichia coli* is a homodimeric class II aaRS (Vincent *et al*., 1995). In most pathogenic bacteria, aaRSs are antimicrobial drug targets for screening and designing enzyme inhibitors, such as the antibiotic pseudomonic acid, an inhibitor of bacterial isoleucyl-rRNA synthetase, which has been used clinically as an agent to prevent *Staphylococcus aureus* infection. aaRS genes may have physiological roles in dealing with harsh environments or be directly involved in biosynthetic pathways (Zeng *et al*., 2009). Godinic-Mikulcic *et al.* (2011) reported that methanogenic archaea can optimize an early translation step by tRNA-synthetase complex under extreme environmental conditions. (4) Molybdenum plays an important role not only in the iron-molybdenum cofactor of nitrogenase, but also in the molybdopterin cofactor of all other molybdoenzymes. *mod* genes (*modA*, *modB*, *modC*, and *modD*) encode a binding-protein-dependent molybdate transport system. *Rhodobacter capsulatus* and *E. coli modD* genes, which are located at a downstream of *modABC* gene, encode a 259-amino acid and 231-amino-acid protein, respectively (Wang *et al*., 1993; Maupin-Furlow *et al*., 1995). Fourfold-higher molybdenum concentrations can inhibit the activity of the alternative nitrogenase in *R. capsulatus modD* mutants, whereas an increase of up to 500-fold is needed for *modC*, *modB*, and *modA* mutants. This indicates that *R. capsulatus* ModD is involved either in molybdenum transport or in signal transduction (Wang *et al*., 1993). Compared to the wild-type strain, a mutation in or deletion of *E. coli modD* gene shows no apparent phenotype difference, suggesting that ModD is not necessary for the molybdate transport system. The *mod* operon is very poorly expressed in the wild-type mod^+ strain, but elevated in a molybdate transport mutant strain or in the wild-type strain growing in a molybdate-deficient medium. This infers that the *mod* operon is repressed in the presence of molybdate. There is no information on the possible physiological functions of ModD protein. The studies of NusA, seryl-tRNA synthetase, and ModD protein mentioned above in *H. pylori* have received little attention thus far.

Taylor *et al.* (1992) speculated that *H. pylori* strains undergo genomic rearrangements to adapt to a new human host environment. The results presented here suggest that *H. pylori* strains express/repress proteins variation, not only in terms of the virulence proteins, but also in terms of physiological proteins when they infect a human host. This raises a question concerning the significance of these proteins in *H. pylori* infection. It is tempting to speculate that these proteins may be involved in disease development during *H. pylori* infection. This linker is predicted to be intrinsically disordered. Disordered proteins may carry out the essential control and regulation functions that are needed to respond to the various environmental conditions, and our analysis of proteins seems to support such hypothesis. If this was the case, it can explain asymptomatic $vacA^+$ $cagA^+$ *H. pylori* carriers during a long bacterial infection. This idea remains to be further tested.

For the two unknown proteins, we speculate that spots appearing well separated on a gel may consist of several proteins. The number of the proteins' mass peaks in peptide mass fingerprinting increased dramatically, such that the spot cannot be identified from an assigned database.

Because the 2-DE technique is too laborious and expensive (Enroth *et al*., 2000), comparative analysis of proteins is, to date, a better method to find a new disease-specific protein antigen (Backert *et al*., 2005). The new specific proteins may be used to find proteins as biomarkers for diagnosis of diseases, and therapy, to monitor diseases associated with *H. pylori* infection. In this preliminary study, we confirm the variation, at the protein level, of *H. pylori* isolated from patients with gastric cancer and gastritis. This reveals completely unexpected complexity and diversity in protein expression. The individual protein expression/repression related to inflammation may be result in the different outcomes of diseases induced by *H. pylori* infection.

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