

Comparative Proteomic Analysis of *Helicobacter pylori* Strains Isolated from Chinese Patients

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ABSTRACT

Helicobacter pylori, the major cause of gastritis, peptic ulcer and gastric cancer, infects half of the world population, but only a few infections lead to serious disease. In order to investigate specific proteins related to the pathogenic difference of this bacterium, comparative proteome analyses of *Helicobacter pylori* C1 (isolated from patients with gastric cancer) and G1 (isolated from patients with gastritis) were performed using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). Four proteins (inorganic pyrophosphatase, 3-oxoadipate CoA-transferase subunit B, translation elongation factor, and aldo-keto reductase) were found only in *Helicobacter pylori* C1, and one protein (alkyl hydroperoxide reductase) was found in G1. Additionally, different isoelectric points (pI) of Hsp60 were observed from the two strains. Then we cloned and sequenced Hsp60 genes from forty-nine *Helicobacter pylori* isolated from gastric cancer and gastritis. Gene sequencing showed that one C→G single nucleotide polymorphism occurred in the 1399th nucleotide of Hsp60. These results indicate that pathogenic differences exist in various *Helicobacter pylori* isolated from Chinese patients.

Keywords: *Helicobacter pylori*; Two-Dimensional Gel Electrophoresis; Proteome Map; Gastric Cancer; Gastritis; Heat Shock Protein 60

1. Introduction

The gastric cancer is the fourth most common cancer and the second leading cause of cancer-related deaths. It is a multistep process caused by multiple factors including human host genotype, physiological, immunological, and environmental factors, as well as *Helicobacter pylori* (*H. pylori*) infection. Epidemiological studies have determined that *H. pylori* confers an attributable risk for gastric cancer of approximately 75% [1]. WHO has ranked *H. pylori* as a class I carcinogen.

H. pylori is a gram-negative, spiral-shaped microaerophilic bacterium that colonizes the human stomach and causes type-B gastritis, gastric/duodenal ulcers, and gastric cancer. According to epidemiological studies, half of the world population is infected by *H. pylori*, but only a fraction of these infections lead to overt disease, including gastric cancer [2]. In addition to the human host

genotype and environmental factors, the *H. pylori* subtype also plays an important role in the outcome of infection.

Strain diversity among *H. pylori* isolates has been studied at the gene level, and some reports have shown that *H. pylori* isolates from different clinical outcomes are genetically diverse with partial virulence factors, which play an important role in pathogenesis [3-6]. With more complete genomes of *H. pylori* strains sequenced [7-10], the information accumulated from genomic studies allows the use of proteomics technologies in studies of these bacteria. Proteomics analyses can be used to study the production and function of proteins coded by active genes and to investigate the pathogenic properties of *H. pylori* strains. Jungblut *et al.* reported that there were certain differences in the positions of protein spots in 2-DE maps of *H. pylori* strains 26695 and J99 [11]. Using comparative analyses of 2-DE maps from *H. pylori* isolates derived from duodenal ulcers and gastritis, Perei-

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ra *et al.* reported that *H. pylori* isolates differentially expressed certain proteins [12]. However, whether gastrointestinal diseases are related to different patterns of *H. pylori* protein expression is still debated and further studies must be performed.

In our study, we compare 2-DE proteome maps of *H. pylori* clinical isolates obtained from patients with gastric cancer and gastritis in order to investigate whether there are specific proteins related to the pathogenic differences.

2. Experimental

2.1. *H. pylori* Strains and Culture Conditions

Forty-nine *H. pylori* strains were isolated from biopsies of gastric antrum mucosa obtained from patients with gastritis (n = 31; No. G1-31; mean age, 53.2 years; male/female, 16/15) and gastric cancer (n = 18; No. C1-18; mean age, 59.8 years; male/female, 10/8). Two of these strains, No. G1 and C1, isolated from the female patients (with age of 48 and 52 years, respectively) at the affiliated hospital of Binzhou Medical University (China), were used to 2-DE. Isolates were grown for 72 h on Columbia agar base, including vancomycin (6 µg/mL), trimethoprim (5 µg/mL), polymyxin B (4 µg/mL), amphotericin B (2.5 µg/mL), and 10% sheep's blood, at 37°C in microaerophilic conditions containing 5% O₂, 10% CO₂, and 85% N₂.

2.2. Sample Preparations

H. pylori strains G1 and C1 were harvested and washed with phosphate-buffered saline (PBS). Total protein was extracted using the trichloroacetic acid (TCA)/acetone precipitation method. Precipitated proteins were lysed overnight at 4°C with lysis buffer, containing 2 M thiourea, 7 M urea, 1% (w/v) DDT, 2% (w/v) CHAPS, 4% immobilized pH gradient (IPG) buffer, and 10 mM PMSF. The suspension was sonicated for 90s on ice, followed by centrifugation for 10 min at 12000 × g. The supernatant was removed and protein concentrations were determined using the Bradford method [13].

2.3. 2-DE and Image Analyses

The first-dimension IEF and second-dimension SDS-PAGE were performed according to the manufacturer's instructions (Pharmacia Biotech, USA). The protein samples (200 µg) were mixed with 450 µL rehydration buffer (8 M urea, 2% CHAPS, 60 mM DTT, and 0.5% IPG buffer) and applied to 24-cm IPG dry strips (pH 3 - 10). Isoelectric focusing was performed for 80,000 Vh over a 25 h period by using the Ettan IPGphor II apparatus (Pharmacia Biotech, USA). The focused strips were equilibrated with equilibration buffer I (50 mM Tris-HCl,

pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 1% DTT) and equilibrated again with equilibration buffer II (50 mM Tris-HCl, pH 8.8, 6M urea, 30% glycerol, 2% SDS, and 4.8% iodoacetamide). After equilibration, SDS-PAGE was performed on 12.5% polyacrylamide gels at 2.5 W for 30 min, and at 18 W until the bromophenol blue reached the bottom of the gel. After staining with Coomassie Brilliant Blue, the individual gels were imaged using an Image Scanner (Pharmacia Biotech, USA) and analyzed by 2-D Image Master V3.1.

2.4. In-Gel Digestion

Differentially expressed spots were excised from the 2-DE gels using a SpotPicker automated gel station (Pharmacia Biotech, USA) and subjected to washing, destaining, reduction, alkylation, trypsin digestion, and peptide extraction. Briefly, each fragment was washed in 25 mM ammonium bicarbonate and destained in a solution of 25 mM ammonium bicarbonate and 30% acetonitrile. After equilibration with 25 mM ammonium bicarbonate, the gel pieces were reduced with 25 mM ammonium bicarbonate (pH 8.0) containing 10 mM DTT for 60 min and alkylated with 25 mM ammonium bicarbonate (pH 8.0) containing 55 mM IAA for 30 min. After equilibration with 25 mM ammonium bicarbonate, each gel fragment was treated with 20 µg/ml trypsin (Sigma), and then incubated in 25 mM ammonium bicarbonate (pH 8.0) at 37°C for 16 h. After trypsinization, the resulting peptides were extracted with 50% ACN and 0.1% TFA, and dried in a vacuum centrifuge. Samples, concentrated to a final volume of 10 µl with 5% TFA in 60% CH₃CN, were used for mass spectrometry analyses.

2.5. Protein Identification by MALDI-TOF-MS Analyses

Peptide analyses were performed using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS). Briefly, protein samples and matrix solution (α -cyano-4-hydroxycinnamic acid prepared in 70% acetonitrile/1.1% trifluoroacetic acid) were mixed (1:1) and loaded into the target wells. The wells were dried at room temperature and subjected to MALDI-TOF-MS analyses using a Reflex (Blaker). MS spectra were analyzed using the program Mascot (based on the NCBIInr and SWISSPROT databases).

2.6. Gene Sequencing

Total DNA was extracted from *H. pylori* strains (G1-31 and C1-18) according to the manufacturer's protocol (QIAGEN, Germany). The Hsp60 gene was cloned using the following conditions: 95°C for 5 min, followed by 30 cycles of 94°C for 30s, 52°C for 30s, and 72°C for 90s.

This was followed by a final extension at 72°C for 5 min. A forward primer (5'-CTTACATCATGCC-3') and a reverse primer (5'-CGTTTCGAAGTAGAGAAATCG-3') were used in this amplification. The products were sequenced by Majorbio Shanghai Technologies Company. Sequence comparisons and analyses were performed using DNASTar 5.0 software.

3. Results

3.1. Protein Expression of *H. pylori* Clinical Isolates

To obtain the protein expression profiles of *H. pylori* G1 and C1, we performed 2-DE analysis. After staining with Coomassie Brilliant Blue, we found that whereas several main spots were in the same position, we observed four spots (spots 1, 2, 3, and 4) of *H. pylori* C1 that were not observed in *H. pylori* G1. There was one spot (spot 5) of *H. pylori* G1 that was not visualized in *H. pylori* C1. In addition, the isoelectric point (pI) of spot 6 is different in the two strains despite its conserved expression. Proteome maps of *H. pylori* G1 and C1 are shown in **Figure 1**.

3.2. Identification of Differentially Expressed Proteins

The differentially expressed protein spots (spot No. 1 - 6) in the different proteome maps were excised from the gel and mass fingerprintings of the 6 proteins were obtained using MALDI-TOF-MS. Corresponding amino acid residue numbers are indicated on the peaks that match the identified protein based on a NCBI database query. Spots numbered 1 - 6 were identified as shown in **Table 1**. In the six proteins, three are enzymes associated with metabolism, including inorganic pyrophosphatase (Ppa), 3-oxoadipate CoA-transferase subunit B (OXCTB), and translation elongation factor (EF-P). Two are related to anti-oxidation effects, aldo-keto reductase and alkyl hydroperoxide reductase (AhpC/TsaA). The final spot, heat shock protein 60, belongs to the chaperonin family.

3.3. Gene Sequencing

The Hsp60 gene was cloned and sequenced. Sequence alignment and analyses were performed using DNASTar 5.0 software. Although the Hsp60 gene contains many highly conserved nucleotide sequences, we found one C→G single nucleotide polymorphism occurred in the 1399th, and 22.58% (7/31) of nucleotides changed from C to G in *H. pylori* strains isolated from patients with gastritis. In one gastric cancer patient, the ratio of nucleotide mutation (C→G) is only 5.56% (1/18) (**Figure 2(a)**). When translated into protein sequences, the nucleotide mutation results in an amino acid substitution at codon 467 from Q (Gln) to E (Glu) (**Figure 2(b)**).

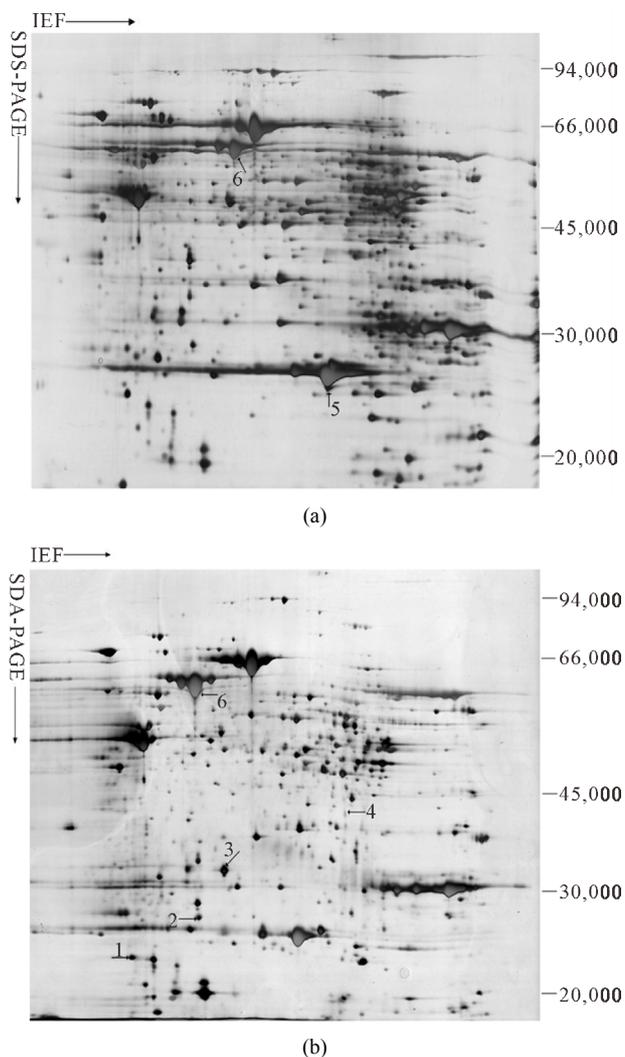


Figure 1. Proteome maps of *H. pylori* clinical isolates G1(a) and C1(b). The protein spots were separated over the molecular weight (Mr) range of 10 - 200 kDa and the pI range of 3 - 10.

4. Discussion

Recently, strain variations in *H. pylori* have been studied at the gene level and compared among different patient groups [3-6]. In this study, we focused on specific *H. pylori* proteins derived from patients with gastric cancer and non-gastric cancer. By comparing 2-DE proteome maps, five proteins were differentially present or absent between *H. pylori* G1 and C1. These proteins are classified into two groups: anti-oxidant proteins and enzymes related to metabolism. The anti-oxidation system, which eliminates damage due to oxygen free radicals released by host macrophages and neutrophils, plays a vital role in *H. pylori* colonization. The energy metabolism of *H. pylori* is essential for its growth and survival. The enzymes related to metabolism are thought to assist virulence factors in modulating adaptation to hosts and to cause per-

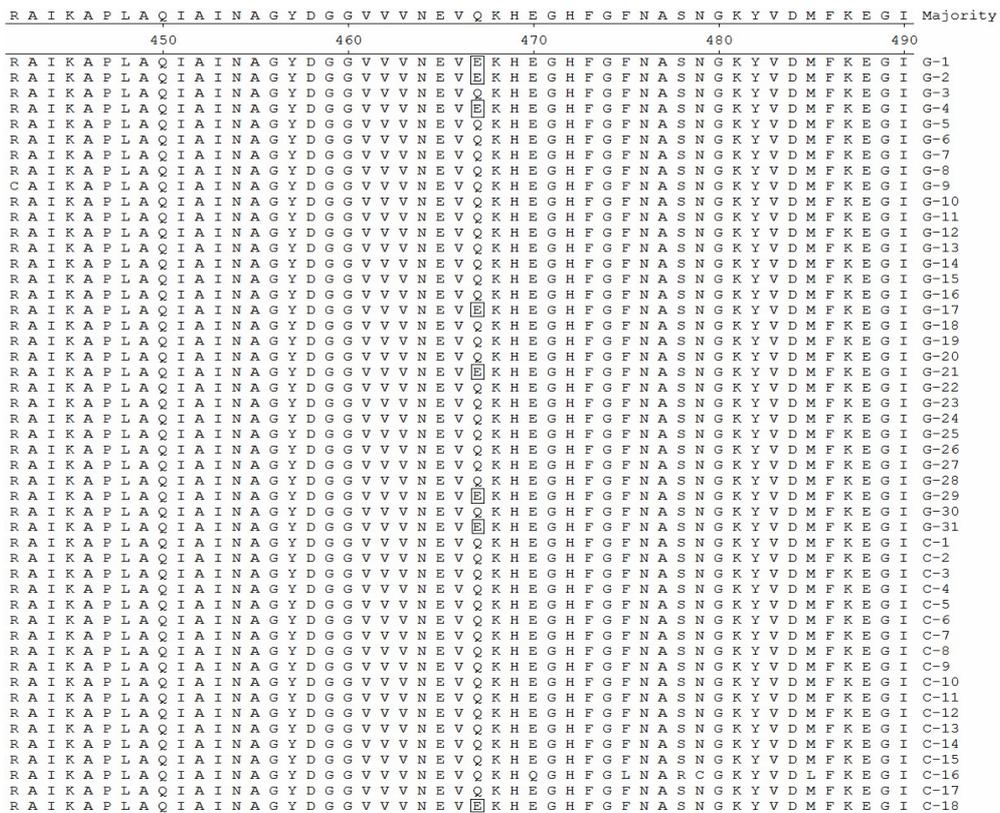
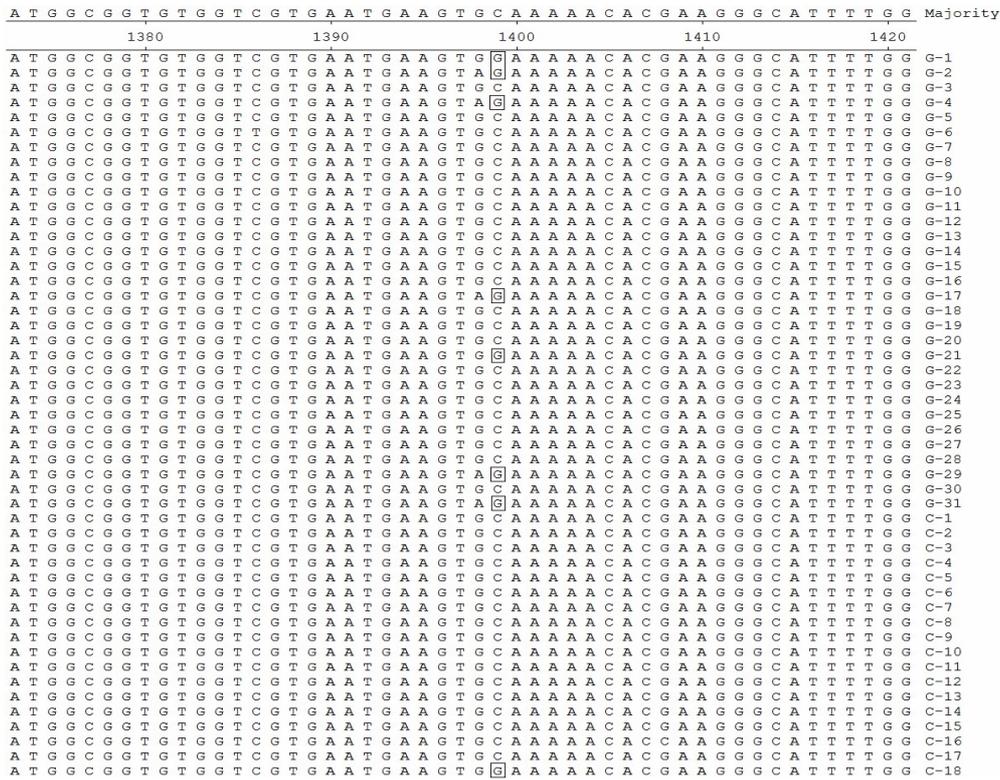


Figure 2. (a) Gene sequence alignment of Hsp60 protein in *H. pylori* clinical isolates; (b) Amino acid sequence alignment of Hsp60 protein in *H. pylori* clinical isolates.

Table 1. Identified proteins on the proteome maps of *H. pylori* isolates.

Spot No.	<i>H. pylori</i> (a)		pI Values	MW (Da)	Identification	Gene (b)
	C1	NC1				
1	+	-	4.80	19271.9	inorganic pyrophosphatase (Ppa)	Hp0620
2	+	-	5.35	22262.9	3-oxoadipate CoA-transferase subunit B (YjxE)	Hp0692
3	+	-	5.35	20787.8	translation elongation factor (EF-P)	Hp0177
4	+	-	7.65	37073.8	aldo-keto reductase	Hp1139
5	-	+	6.25	22235.6	alkyl hydroperoxide reductase (AhpC/tsaA)	Hp1563
6	+	+	5.55	58227.5	chaperone and heat shock protein (GroEL)	Hp0010

(a) Symbols “+” or “-” show the presence or absence of the protein spot on the proteome maps, respectively; (b) Homolog of the gene of *H. pylori* strain 26695.

sistent bacterial infection. Combining our results with other pertinent studies [11,14-16], these strain-specific proteins are thought to play a role in the pathogenicity of *H. pylori* and can be used for *H. pylori* infection characterization. However, this hypothesis is still debated and requires more studies.

In addition, differences in pI values of Hsp60 were found in our study. The Hsp60 spot from *H. pylori* C1 isolated from gastric cancer shifted into acidic regions compared to the *H. pylori* G1 isolated from non-gastric cancer, despite its conserved expression. Interestingly, our results are similar to what has been previously reported. Govorun *et al.* characterized some proteins of *H. pylori* isolates, including Hsp60, by different pI values [12,17]. Krahl *et al.* also reported that the pattern expression of Hsp60 was different in gastrointestinal diseases [18]. Many reports have shown that Hsp60 is an immunogen and can lead to host inflammation [19-22]. Hsp60, which has different pI values, may play a different role in inflammation and result in different diseases induced by *H. pylori* infection.

We speculated that these changes in pI values might have been attributed to possible amino acid mutations. Therefore, we cloned and sequenced the Hsp60 gene. At the 1399th, we found the ratio of nucleotide mutation (C→G) was different in *H. pylori* associated with gastric cancer and non-gastric cancer (5.56% vs. 22.58%). With the increasing availability of gene sequences, sequence analyses of Hsp60 genes have been reported. It can distinguish species and/or subspecies in different taxa, such as *Enterococcus* [23,24], *Staphylococcus* [25], and *Bifidobacterium* [26]. Therefore, the utility of Hsp60 for bacterial species identification is well established. The 1399th site mutation of Hsp60 might be a biomarker for identification of *H. pylori* associated with gastric cancer.

In this study, we have investigated variations in different *H. pylori* isolates at the protein level and found differently expressed protein spots that are useful for characterizing *H. pylori* strains. A single nucleotide polymorphism at the 1399th of Hsp60 might be a biomarker for distinguishing subspecies of *H. pylori* strains but this hypothesis requires more research.

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