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Comparative Proteomics Analysis of Sarcosine Insoluble Outer Membrane Proteins from Clarithromycin Resistant and Sensitive Strains of *Helicobacter pylori*[§]

Rebecca Smiley[1], James Bailey[2], Mahadevan Sethuraman[2], Norberto Posecion[2], and M. Showkat Ali[1,2*]

[1]*Department of Clinical Investigation, William Beaumont Army Medical Center, 5005 Piedras Street, El Paso, TX 79920-5001, USA*
[2]*Texas Tech Health Science Center Paul L. Foster School of Medicine, 5001 El Paso Drive, El Paso, TX 79905-2709, USA*

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Helicobacter pylori **causes disease manifestations in humans including chronic gastric and peptic ulcers, gastric cancer, and lymphoid tissue lymphoma. Increasing rates of *H. pylori* clarithromycin resistance has led to higher rates of disease development. Because antibiotic resistance involves modifications of outer membrane proteins (OMP) in other Gram-negative bacteria, this study focuses on identification of *H. pylori* OMP's using comparative proteomic analyses of clarithromycin-susceptible and -resistant *H. pylori* strains. Comparative proteomics analyses of isolated sarcosine-insoluble OMP fractions from clarithromycin-susceptible and -resistant *H. pylori* strains were performed by 1) one dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis protein separation and 2) in-gel digestion of the isolated proteins and mass spectrometry analysis by Matrix Assisted Laser Desorption Ionization-tandem mass spectrometry. Iron-regulated membrane protein, UreaseB, EF-Tu, and putative OMP were down-regulated; HopT (BabB) transmembrane protein, HofC, and OMP31 were up-regulated in clarithromycin-resistant *H. pylori*. Western blotting and real time PCR, respectively, validated UreaseB subunit and EF-Tu changes at the protein level, and mRNA expression of HofC and HopT. This limited proteomic study provides evidence that alteration of the outer membrane proteins' profile may be a novel mechanism involved in clarithromycin resistance in *H. pylori*.**

Keywords: outer membrane protein, clarithromycin, antibiotic resistance

*For correspondence. E-mail: Mohammed.S.Ali@amedd.army.mil; Tel.: +1-915-742-6041
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Introduction

Helicobacter pylori infection has been linked to development of chronic gastritis, gastric and duodenal ulcers, gastric adenocarcinoma, and lymphoid tissue lymphoma (Anderson *et al*., 2008; Hatakeyama, 2009). *H. pylori* infection can result in a predictive sequence of events leading to the development of gastric cancer. Gastric cancer development due to *H. pylori* infection involves both direct and indirect pathways. The direct pathways involve protein modulations (Xia *et al*., 2008; Axsen *et al*., 2009), deregulation of signaling pathways (Muller *et al*., 2011), gene mutation (Huang *et al*., 2011; Toller *et al*., 2011), epigenetic changes and chromosomal alterations (Katayama *et al*., 2009), and production of reactive oxygen species (Katsurahara *et al*., 2009). Indirectly, *H. pylori* induces an inflammatory response: infiltration of T cells and activated mononuclear cells which produce an enhanced production of a variety of pro-inflammatory cytokines in the gastric mucosa (Li *et al*., 2008a; Isomoto *et al*., 2012).

While 60–80% of gastric cancers are linked to *H. pylori* infection in the developing world, only 1–2% are *H. pylori*-linked in western countries (Hunt *et al*., 2011). Because of *H. pylori*'s predilection for gastric cancer development, early eradication of infection is imperative. Current treatment of *H. pylori* infection includes the use of antimicrobials, a proton-pump inhibitor (PPI), and in some instances a bismuth compound. This triple therapy has classically included the use of clarithromycin together with either amoxicillin or metronidazole, and a PPI for 7–14 days. Clarithromycin is the most effective single drug used and leads to an eradication rate of approximately 40% when given twice daily for 10–14 days. However, reports indicate that nearly 25% of the treatment failures are due to clarithromycin resistance (Wu *et al*., 2012), and decreases in eradication rates of 70% or more have been observed in infections involving clarithromycin-resistant strains (Duckworth *et al*., 2009; Boyanova and Mitov, 2010). Furthermore, the natural competence of *H. pylori* makes the spread of clarithromycin resistance even more worrisome, as genetic material exchanges easily between *H. pylori* bacteria (Dorer *et al*., 2010).

In Gram-negative bacteria, outer membrane proteins (OMPs) are known to be involved in antibiotic resistance. For example, loss of expression of OMPs plays a vital role in the development of antibiotic resistance in *Pseudomonas aeruginosa*, *E. coli*, and *Vibrio cholera* (Delcour, 2009). The *H. pylori* genome is predicted to encode 32 different OMP genes which are divided into five groups (Cover, 2006). The largest family includes adhesins. The other families include porins,

iron transporters, efflux proteins, and proteins of unknown function. *H. pylori* OMPs have not been as thoroughly investigated and more research needs to be conducted to elucidate the role of these proteins in pathogenesis, antibiotic resistance, their relation to other virulence factors, and their potential as antigens in vaccine development.

Thus, our specific aim was to identify the OMP(s) of *H. pylori* that contribute to antibiotic resistance using a comparative proteomic analysis of clarithromycin-susceptible and -resistant *H. pylori* strains. This was achieved by one dimensional SDS-PAGE followed by mass spectrometry identifications of in-gel digested proteins of interest. Delineation of the mechanism of antibiotic resistance will not only help to eradicate *H. pylori* infection, but may also provide mechanistic insights into gastric cancer, the second most common cause of cancer death worldwide.

Materials and Methods

Bacterial strains and cultural conditions

H. pylori clarithromycin susceptible (ATCC no. 43504) and resistant strain (ATCC no. 700684) from American Type Culture Collection (USA) were used in this study. The bacteria were cultured on blood agar plates containing 5% sheep blood at 37°C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) using CampyPack pouches (Fisher Scientific, USA) for 72–84 h. The strains were verified by Gram staining, documenting the cell morphology, and testing for catalase, oxidase and urease activity. Clarithromycin susceptibility was verified using a disc diffusion methodology employing 5% sheep blood agar plates (Tanih *et al*., 2010).

Isolation of sarcosine insoluble outer membrane proteins

The bacterial strains were grown as described above, harvested by centrifugation (5,000×*g*, 15 min, 4°C), and washed three times with 20 mM Tris-HCl (pH 7.5). The cells were then briefly homogenized in ice-cold hypotonic buffer [20 mM Tris-HCl; pH 7.5, 1.0 mM $MgCl_2$, 1 mM EDTA, and 1× protease inhibitor cocktail (Sigma Aldrich, USA)] using an ultrasonicator. Lysates were centrifuged twice, first at 5,000×*g* (10 min, 4°C) to remove unbroken cells and then at 40,000×*g* (30 min, 4°C) to precipitate the total plasma membrane. The resulting total membrane preparation was resuspended in the same hypotonic buffer, supplemented with 2% sodium lauryl sarcosine, and then incubated at room temperature for 1 h. The sarcosine insoluble OMPs of *H. pylori* were then collected by centrifugation as described previously (Baik *et al*., 2004). The protein concentration of the OMP preparation was determined using a protein assay kit available from Bio-Rad (USA).

SDS-polyacrylamide gel electrophoresis

Equal amounts of OMPs (10 μg) from sensitive and resistant strains were separated on a 10% SDS-PAGE (1-D-SDS-PAGE). Protein bands in the gel were detected either by Coomassie brilliant blue (CBB) R-250 or silver stain kit (Thermo Scientific, USA). The level of expression of OMPs between resistant and sensitive strains was quantitated using Li-COR densitometric analysis software.

In-gel digesion, MALDI-TOF TOF mass spectrometry and database searching

The stained protein band of interest was excised from the 1D gels and digested with trypsin using a modified version of the protocol developed by Shevchenko *et al*. (1996). Briefly, excised gel bands were cut into small pieces and destained in 0.1 M NH_4HCO_3, partially dehydrated with 50% acetonitrile, followed by complete dehydration with acetonitrile, and dried using Savant (Thermofisher, USA) Speed-Vac concentrator. The alkylation and reduction of proteins were done using 25 mM DTT and 55 mM iodoacetamide solutions. The gel pieces were then rehydrated with 12.5 ng/μl trypsin (Promega, USA) solution (in 0.01% ProteaseMax surfactant in 50 mM NH_4HCO_3) and incubated for 1 h at 50°C. Peptides were extracted from the gel pieces and desalted using ZipTip (Millipore, USA). The desalted peptides were spotted onto an Opti-TOF^{TM} 384 well insert (123×81 mm) (Applied Biosystems, USA) with 0.5 μl 10 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics) as the matrix. The digests were analyzed using the AB SCIEX 5800 TOF TOF in reflector mode for positive ion detection. Protein identification by a combined MS and MS/MS spectral data search was performed against the *H. pylori* protein sequence database (deduced from UniProt) using Paragon (V.4.0.0.0, Applied Biosystems) algorithm in ProteinPilot software (Beta 4.1.46, AB SCIEX, USA) with a detected protein threshold of (>0.47 in unused ProtScore) >95% confidence.

Quantitative RT-PCR (qPCR) analysis

Real-time PCR was used to quantitate the mRNA level of HofC in clarithromycin-resistant and -susceptible strains. Briefly, DNA-free total RNA was isolated from both bacterial strains using an RNeasy mini kit (Qiagen Sciences, USA) according to the manufacturer's instructions. Subsequently, cDNA was reverse transcribed from 2 μg total RNA and 1 μg random hexamers according to the manufacturer's protocol (Life Technologies, USA).

qPCR was carried out on an Applied Biosystems 7900 HT Real-Time PCR Instrument. Each reaction mixture consisted of cDNA, forward and reverse primers, AmpliTaq Gold master mix and nuclease free water in a total volume of 10 μl. qPCR was carried out according to the following amplification protocol: 50°C for 2 min, 95°C for 10 min, 45 cycles of (5°C for 15 sec, 60°C for 1 min) followed by a standard dissociation (95°C, 60°C, 95°C). During PCR cycling fluorescence data was acquired during the 60°C step; during the dissociation fluorescence data was acquired continuously between 60°C and 95°C. Transcript levels were determined by absolute quantification (standard curve method), and then were normalized to the amount of 16S RNA per sample. Primers for HofC and HopT and 16S rRNA were designed to have a melting temperature (T_m) between 58°C and 60°C, and to generate an amplicon between 70 and 100 bp in length (The primer sequences are described on Supplementary data Table S1). The primers were then tested for specificity *in silico*, using NCBI's Blastn program, and *in vitro*, by analyzing dissociation curves generated with genomic DNA.

Assays were performed using RNA isolated from three independent experiments. For each assay sextuplet reactions were set with each sample and with non template control.

Western blot analysis of Urease B subunit and elongation factor Tu

OMPs were resolved using electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Approximately 2 and 10 μg of OMPs were loaded into separate wells from sensitive and resistant strains to detect UreaseB subunit and Elongation factor Tu (EF-Tu) respectively. The membrane was incubated in blocking buffer [5% nonfat dry milk in Tris buffer saline including 0.1% Tween 20 (TBST-5% milk)] for 3 h at room temperature. After blocking, the membrane was incubated at room temperature with a Urease B subunit polyclonal antibody (anti-Urease pAb) at a dilution of 1:5000 for 4 h. This antibody specifically recognizes the Urease B subunit isoform (AbCam, USA). To detect the EF-Tu, the membrane was then incubated with EF-Tu monoclonal antibody (anti-EF-Tu mAb) obtained from Hycult Biotech (Plymouth Meeting, USA) at a dilution of 1:100. After primary antibody incubations, the membranes were then washed in TBST-5% milk four times for 15 min each and then incubated in diluted (1:20,000) IRDye800-conjugated anti-rabbit secondary antibody for 45 min at room temperature. The membranes were then washed four times for 15 min each with TBST, and a digital image of the blot was captured using the Odyssey™ Infrared Imaging system (LI-COR Biosciences, USA). The relative intensity of the UreaseB subunit and EF-Tu protein band was quantified using Li-COR densitometric analysis software.

Results and Discussion

Isolation and characterization of outer membrane proteins

In this study we used sarcosine insoluble fractionation to isolate the OMPs from clarithromycin-susceptible and -resistant *H. pylori* strains. This method has already been adapted to *H. pylori* previously (Doig and Trust, 1994; Baik *et al.*, 2004) and is well established in other bacterial systems (Li *et al.*, 2008b). Subsequent identification of protein species was achieved by one dimensional SDS-PAGE (1-D-SDS-PAGE) and peptide mass fingerprinting (PMF) using MALDI-TOF-TOF MS. The results described in Fig. 1 show that the sarcosine-insoluble fraction contained at least twelve major proteins, and that their molecular masses were between approximately 20 and 100 kDa, as determined by 1-D-SDS-PAGE and CBB staining. SDS-PAGE was visualized by silver staining for more sensitive protein detection, which revealed a protein pattern consistent with CBB staining. We found that a few proteins were in low abundance and excluded from MALDI-TOF-TOF MS analysis. Despite the fact that *H. pylori* is reportedly a very genetically diverse organism, the separation of OMPs revealed an almost identical pro-

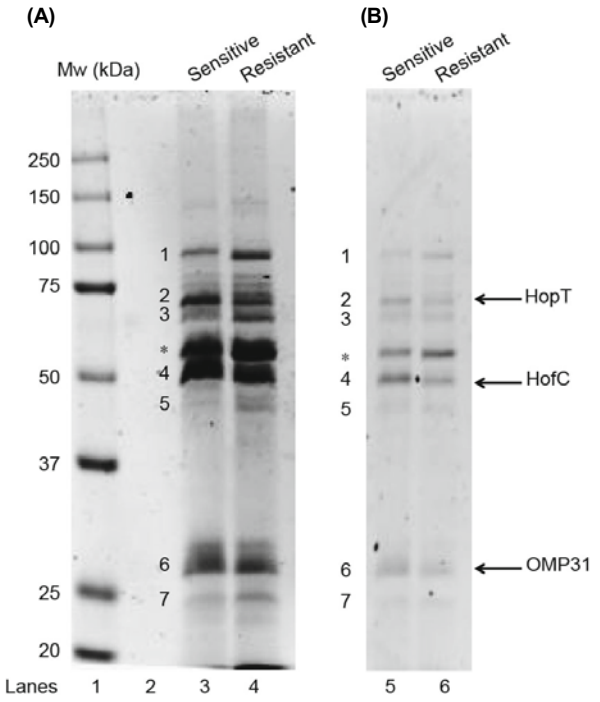

Fig. 1. Differential OMPs profile of Clariothromycin resistant and sensitive strains of *H. pylori*. (A) Ten microgram of OMPs were separated on 10% SDS-PAGE and CBB stained. Lanes: 1, molecular weight marker; 2, empty lane; 3, resistant strain; 4, sensitive strain. Molecular marker on the left was used to calculate the relative molecular masses of OMPs. Out of the twelve proteins, seven showed differential expression change. The seven differential protein bands were identified by MALDI-TOF-TOF MS and belonged to iron regulated membrane protein (band-1), HopT (band-2), UreaseB (band-3), HofC (band-4) elongation factor Tu [(EF-Tu) band-5], and, OMP31 (band-6), putative OMP (band-7). The results represent from three separate gels that were electrophoresed under identical conditions. HopT, (band-2), HofC (band-4), OMP31 (band-6) and an unidentified protein marked with asterisk were overloaded. (B) The differential expression of HopT, HofC, OMP31 and an unidentified protein (marked with an asterisk) was further confirmed by separating low amounts of protein (1 μg) via SDS-PAGE followed by CBB staining. The experimental conditions were the same as (A).

Table 1. Identification of altered OMP expression in resistant and sensitive strains of *H. pylori*[a]

Band No.	Acession No.	Sequence coverage (%)	Peptide matched	Name
1	E6S3E6_HELPF	26	9	Iron-regulated outer membrane protein
2	B6JN83_HELP2	32.5	9	HopT/BabB
3	E8QRG4_HELPR	28.7	5	UreaseB subunit
4	D0JZ01_HELP5	30.3	12	HofC
5	G2MAL4_HELPX	20.1	2	Elongation factor Tu (EF-Tu)
6	D6XS18_HELPV	44	6	Outer membrane protein31 (OMP31)
7	Q9ZM01_HELPJ	49.05	3	Putatative outer membrane protein

[a] Proteins were identified by MALDI-TOF-TOF MS of a single 1-D-SDS-PAGE. Protein identity is significant at the 95% level.

tein profile, but with different levels of protein expression, between resistant and sensitive strains (Figs. 1A and 1B).

Out of 12 proteins, the seven proteins that showed the most significant difference in expression were numbered, excised, destained, in-gel digested with trypsin and subjected to MALDI-TOF-TOF analysis. Table 1 indicates the identities of these proteins, which were obtained from PMF and data base search. Representative MS/MS spectra of peptides for some of the proteins identified in the database search with >95% confidence are shown along with the matching b and y fragment ions in the Supplementary data (Fig. S1). The majority of the OMPs identified in this study have been reported to be surface expressed proteins. In addition, protein identity was significant at the 95% level. We found that four proteins were down-regulated and three proteins were up-regulated with a fold difference ≥2 and a statistical significance of $P<0.05$, as quantified by densitometric analysis (data not shown), in the resistant strain. Iron-regulated membrane protein (band-1), UreaseB (band-3), elongation factor Tu [(EF-Tu) band-5], and putative OMP (band-7) were down-regulated, whereas transmembrane protein HopT (band-2), HofC (band-4), and OMP31 (band-6) were up-regulated. As can be seen in Fig. 1, total amount of HopT, HofC, OMP31, and an unidentified protein (marked with an asterisk) were overloaded and therefore difficult to confirm their expression levels. By separating low amounts of protein (1 μg) following SDS-PAGE, we were, however, able to confirm the expression levels. Interestingly, there was a substantial difference in the levels of HopT, HofC, and OMP31 expression (Fig. 1B). Although the unidentified protein* tended to be reduced in the resistant strain (Fig. 1B, compare lane 5 with lane 6), the expression level was not statistically significant as determined from three independent experiments and was excluded from MALDI-TOF-TOF MS analysis.

The *H. pylori* genome is predicted to encode thirty two different OMP genes (Cover, 2006), whereas the number of major OMPs identified in this study was twelve, significantly lower. This could be explained by the fact that the synthesis of OMPs depends on culture conditions, and several OMPs are synthesized exclusively during interactions with host cells. Although there are no reports describing the comparative proteomics of antibiotic resistant and susceptible strains, there are a few reports of isolation of OMPs from antibiotic-susceptible strains of *H. pylori*. These reports indicate that the number of proteins identified varies from preparation to preparation. For example, Doig *et al.* (1994) identified six OMPs, while Baik *et al.* (2004) identified sixteen OMPs in the sarcosine insoluble fraction from the same *H. pylori* 26695 strain. Another study reported fourteen OMPs including eight newly detected proteins expressed in *H. pylori* J99 (Park *et al.*, 2008).

Comparison of the major proteome components between resistant and sensitive strains

Iron is a micro-nutrient essential for the survival, growth, and expression of virulence factors in *H. pylori*. The genome sequence of *H. pylori* suggests that this bacterium possesses eleven proteins predicted to be involved in iron transport and two proteins thought to function as iron storage proteins (van Vliet *et al.*, 2001). Among these proteins, six of them were predicted to be iron-regulated OMPs and subdivided into FecA-like and FrpB-like proteins (Velayudhan *et al.*, 2000; van Vliet *et al.*, 2002). In our study the identified iron-regulated protein of approximately 98 kDa molecular mass (Fig. 1, band-1) coincides with the sequence of the FecA-3 iron protein (Thiberge *et al.*, 2010). Many of these iron-regulated OMPs have been reported to be involved in *H. pylori* pathogenesis but the role of these proteins in development of antibiotic resistance remains unknown.

The largest family of OMPs includes Hop proteins or adhesion proteins and enables *H. pylori* to attach and adhere to gastric epithelial cells. Available evidence suggests that expression of Hop proteins is associated with severe gastroduodenal disease (Yamaoka *et al.*, 2006). At least twenty-one different Hop proteins have been identified and two of them are reported to be porin proteins (Alm *et al.*, 2000). The other known functions of Hop proteins include neutrophil infiltration, induction of IL-8 and gastric mucosa damage (Loh *et al.*, 2008). Our results show that there is increased expression of HopT (*BabB*) in the resistant strain compared to the sensitive strain (Figs. 1A and 1B, band-2), implying a poorer clinical outcome and/or increased survival of resistant strains. We also evaluated the expression level of the gene encoding HopT in the sensitive and resistant strains via qPCR using an absolute quantification method. Figure 2A indicates that there is poor correlation between HopT mRNA transcript levels and protein expression in the resistant strain, suggesting that mRNA and protein stability may be affecting proteomic and transcriptional profiling. Notably, we could not detect the expression of *BabA*, a well

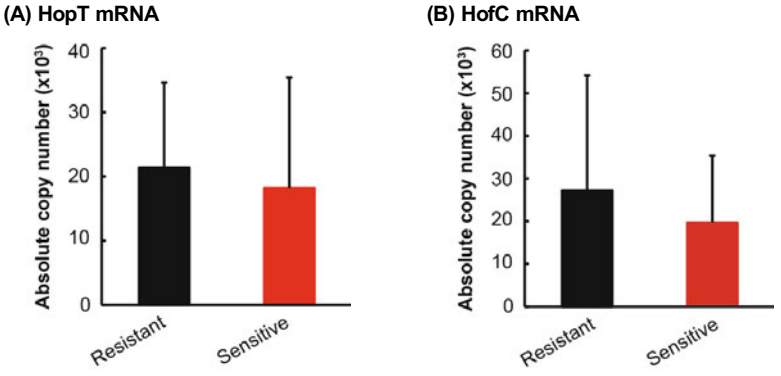

Fig. 2. Expression of HopT (A) and HofC (B) mRNA in resistant and sensitive strains of *H. pylori*. cDNA was prepared following standard procedure; qPCR was performed using AmpliTaq Gold master mix as described in 'Materials and Methods' section. Assay performance was evaluated by constructing a standard curve for each mRNA as well as negative control. The values are reported as the averages from three independent assays. Samples were normalized to the levels of 16S rRNA.

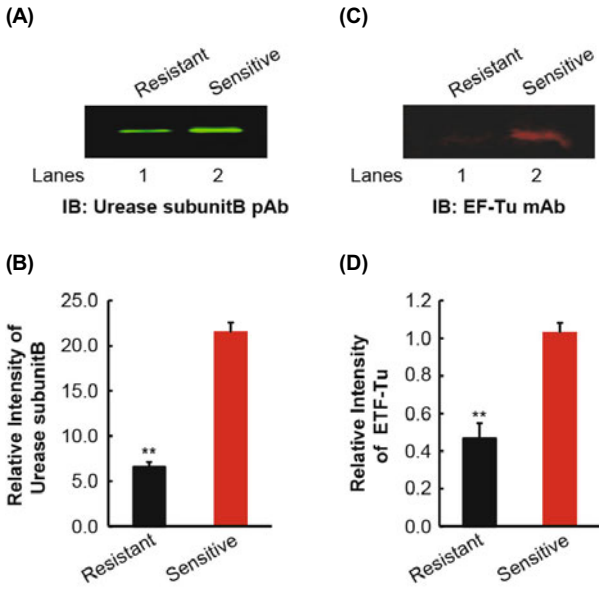

Fig. 3. Level of UreaseB and EF-Tu in resistant and sensitive strains of *H. pylori*. To investigate the differential expression of UreaseB and EF-Tu, outer membrane preparations containing equal amounts of protein were loaded onto each lane. The proteins were transferred to a nitrocellulose membrane and western blotted with the indicated primary antibodies followed by incubation with goat IRDye 800-conjugated anti-rabbit or anti-mouse secondary antibody, and the image was captured using the Odyssey™ Infrared Imaging system. The relative intensity of the UreaseB subunit and EF-Tu protein band was quantified using Li-COR densitometric analysis software. The data are from three repeats. (A) Representative immunoblot showing the level of expression of UreaseB in resistant and sensitive strains of *H. pylori.* (B) Quantitative analysis of the immunblot expressed as arbitrary units showing the significant difference in level of expression between sensitive and resistant strains. $^{**}P<0.001$. (C) Representative immunoblot showing the level of expression of EF-Tu in resistant and sensitive strains of *H. pylori.* (D) Quantitative analysis of the immunblot showing expressed as arbitrary units the significant difference in level of expression between sensitive and resistant strain. $^{**}P<0.001$.

characterized adhesion protein, in our outer membrane preparation. It has previously been reported that in some strains of *H. pylori*, the *babA* gene is replaced by *babB*/hopT, whereas in other strains the *babA* gene is present but is not expressed because of alteration in dinucleotide CT repeats in the 5' coding region (Hennig *et al.*, 2004). Based on this evidence, we hypothesize that *babA* may not be expressed in these strains, or that a gene conversion might make it possible to identify predominantly *babB/hopT*. *BabA* is one of the best characterized adhesin proteins and is known to facilitate colonization and induce mucosal inflammation by adhering to gastric epithelial cells via Lewis B blood group antigens (Shao *et al.*, 2005). Given that *hopT*(*babB*) is a gene homologue of *babA, hopT* might serve as a potential target for drug discovery and vaccine development.

HofC is a surface protein present in both clarithromycin-resistant and sensitive strains, but upregulated in resistant strains (Figs. 1A and 1B, band-4). qPCR techniques were utilized to measure cellular HofC mRNA levels. We found that the high level of expression of HofC in the resistant strain, as determined by 1-D-SDS-PAGE, was consistent with the 2-fold increase in mRNA abundance for this gene (Fig. 2B). Although HofC in the resistant strain tended toward increased mRNA expression, the results were not statistically significant ($P<0.23$). However, quantitaion of protein (band-4) from three independent experiments using Li-COR densitometric analysis software showed significant difference between resistant and sensitive strains (data not shown). It is noteworthy to mention that divergence between gene and protein expression findings is typical and can be explained as mRNA expression levels do not guarantee that a specific mRNA is properly translated, folded, and trafficked to produce functional protein. HofC is a member of a paralogous Hof family that contains eight members (Alm *et al.*, 2000). Apart from the fact that HofC is non-heat modifiable (Exner *et al.*, 1995; Alm *et al.*, 2000), nothing is known about its function. Studies are needed to determine whether increased HofC expression in resistant strains contribute to the mechanism involved in decreased susceptibility to clarithromycin.

Several investigations have suggested a role for *H. pylori* urease in the survival and pathogenesis of the bacteria. The urease gene encodes two primary structural proteins: UreaseA and UreaseB. Of these proteins, we could detect only UreaseB in our outer membrane preparation and found that UreaseB expression levels were significantly decreased in the resistant strain as determined by western blotting (Fig. 3A, compare lane 1 with lane 2 in upper panel; Fig. 3B, 6.57±0.58 vs 21.48 ±0.58 1.11; $^{**}P<0.001$). Although UreaseB is typically a cytoplasmic protein, the unique structural organization of the *H. pylori* outer membrane allows for the incorporation of many other cytoplasmic proteins, including catalase, superoxide mutase and urease (Phadnis *et al.*, 1996). Thus, the presence of UreaseB in the OMP preparation is not a cytoplasmic contaminant, as was observed by many other investigators (Phadnis *et al.*, 1996; Schoep *et al.*, 2010) but a true surface protein. Electron microscopic studies have also revealed that urease is located both in the cytoplasm and in the outer membrane (Keenan *et al.*, 2000; Mullaney *et al.*, 2009), supporting our observations. The enzymatic activity of urease neutralizes stomach acidity, thereby promoting infection by this pathogen. Additional urease functions include enhancement of bacterial motility, defense of the bacteria from $ONOO^-$ cytotoxicity and inhibition of phagocytic clearance of bacteria (Dhar *et al.*, 2003). The functional significance of the differential expression of UreaseB between resistant and sensitive strains demonstrated in this study is unclear and deserves further investigation.

EF-Tu, which is encoded by *tuf* genes, is associated with the cytoplasmic membranes of Gram-positive bacteria and the outer membranes of Gram-negative bacteria (Wilkins *et al.*, 2003; Defeu Soufo *et al.*, 2010). The observed differential expression of EF-Tu in the OMP preparation from SDS-PAGE and mass spectrometry results were confirmed by western blot analysis. Equal amounts (10 µg) of outer membrane preparation from resistant and sensitive strain were loaded onto each lane detected with anti-EF-Tu mAb. As shown in Fig. 3C (compare lane 1 with lane 2), expression of EF-Tu was down-regulated in the resistant strain. The densitometric analysis of the immunoblot revealed that there is a two-fold difference in level of expression of Ef-Tu between resistant and sensitive strain (Fig. 3D, 0.47±0.078 vs 1.028±0.053, $^{**}P<0.001$). Although EF-Tu primarily acts as an elongation factor during protein translation, it has pre-

viously been demonstrated that EF-Tu also plays a role as a virulence factor in Gram-negative bacteria. For example, surface-translocated EF-Tu mediates binding to fibronectin and other host proteins in *Mycoplasma pneumonia* (Dallo *et al*., 2002) and *Pseudomonas aeruginosa* (Kunert *et al*., 2007), contributing to their virulence. Reduced expression of EF-Tu in clarithromycin resistant *H. pylori* strains might suggest reduced virulence, if it is shown that EF-Tu is, in fact, a virulence factor in *H. pylori*.

The expression of OMP31 was up-regulated in the resistant strain (Figs. 1A and 1B, band-6). Doig and Trust (1994) reported that the highly conserved OMP31 protein belongs to the porin family. Although OMP31 is presumed to be a porin, no specific function has been demonstrated. Information about a potential role for putative OMP (band-7) in *H. pylori* is lacking and identification of homologous proteins in other bacteria remains to be determined.

There are two major limitations of our study that need to be addressed. First, our use of 1-D-SDS-PAGE followed by MALDI-TOF-TOF MS may limit the identification of proteins co-migrating on the gel. However, it is well known that two dimensional gel electrophoresis (2-DE) is not the method of choice for hydrophobic and low-abundance proteins (i.e. OMPs). Considering these limitations of 2-DE, we believe that the 1-D-SDS-PAGE reference map of the OMP subproteome of *H. pylori* established in this study can serve as a platform for future investigations in the field of antimicrobial resistance. Second, the functional significance of the proteins identified in this study in relation to antibiotic resistance remains to be determined, and such work is beyond the scope of this study.

We identified seven differentially expressed proteins characterized as iron regulatory proteins, adhesion proteins, host-pathogen interaction proteins, and hypothetical proteins of unknown function. Together, these proteins might synergistically give a survival advantage to resistant *H. pylori* in the presence of clarithromycin. This study utilizes a rapid subproteomic approach to characterize OMPs, and highlights a novel antibiotic resistance mechanism in *H. pylori* which involves the alteration of expression levels of OMPs. Future efforts may provide insights into universal OMP targets for the development of new drugs that will control antibiotic resistance in this organism. Furthermore, identified OMPs might serve as biomarkers for multi-drug resistance in clinical specimens.

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Authors' contribution: RS conceived and performed the experiments; JB made contributions to the project design and coordinated the study; MS and NP performed the proteomics sample preparation and mass spectrometry analysis; MSA conceptualized the study, analyzed the data and wrote the manuscript.

Competing interests: The authors have no competing interests.

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