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Alteration of the *Helicobacter pylori* membrane proteome in response to changes in environmental salt concentration

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Abstract

Purpose—*Helicobacter pylori* infection and a high dietary salt intake are each risk factors for the development of gastric cancer. We hypothesize that changes in environmental salt concentrations lead to alterations in the *H. pylori* membrane proteome.

Experimental Design—Label-free and iTRAQ methods were used to identify *H. pylori* proteins that change in abundance in response to alterations in environmental salt concentrations. In addition, we biotinylated intact bacteria that were grown under high- or low-salt conditions, and thereby analyzed salt-induced changes in the abundance of surface-exposed proteins.

Results—Proteins with increased abundance in response to high salt conditions included CagA, the outer membrane protein HopQ, and fibronectin domain-containing protein HP0746. Proteins with increased abundance in response to low salt conditions included VacA, two VacA-like proteins (ImaA and FaaA), outer-membrane iron transporter FecA3, and several proteins involved in flagellar activity. Consistent with the proteomic data, bacteria grown in high salt conditions exhibited decreased motility compared to bacteria grown in lower salt conditions.

Conclusions and clinical relevance—Alterations in the *H. pylori* membrane proteome in response to high salt conditions may contribute to the increased risk of gastric cancer associated with a high salt diet.

Keywords

Proteomics; mass spectrometry; gastric cancer; motility; outer membrane proteins

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Introduction

About 50% of humans are persistently colonized by *Helicobacter pylori* [1, 2]. While most colonized individuals remain asymptomatic, a small proportion develop severe disease manifestations, including peptic ulceration, gastric adenocarcinoma, or mucosa associated lymphoid tissue (MALT) lymphoma [1–3]. Multiple risk factors for the development of symptomatic disease have been identified. These include infection with *H. pylori* strains that produce strain-specific virulence factors, such as CagA, s1m1-type VacA, and type 1 HopQ [4–6], as well as host genetic factors and certain environmental conditions. For example, high dietary salt intake is associated with an increased risk of gastric cancer [7–10].

H. pylori CagA is translocated into gastric epithelial cells via the *cag* PAI-encoded type IV secretion system and causes numerous changes within the cells [6, 11]. Studies in animal models have shown that CagA contributes to *H. pylori*-induced gastritis and has an important role in the development of gastric cancer [12–14]. *In vitro* studies have demonstrated an increased production of CagA in *H. pylori* grown under high salt conditions, compared to bacteria grown under low salt conditions [15, 16]. Therefore, it is hypothesized that salt-induced alterations in CagA production contribute to the increased gastric cancer risk associated with a high salt diet [10, 12].

Surface-exposed outer membrane proteins also have an important role in mediating interactions between *H. pylori* and host cells. For example, surface-exposed *H. pylori* proteins can function as adhesins [17, 18], and can influence the severity of gastric mucosal inflammatory responses [19, 20]. Thus far, there has been relatively little progress in analyzing the regulation of *H. pylori* membrane protein production in response to changes in environmental salt concentration. A previous study used 2D-DIGE proteomic methods to analyze *H. pylori* lysates and identified 31 *H. pylori* proteins that are regulated in response to changes in the salt concentration of the bacteriologic culture medium [15], but 2D-DIGE is considered suboptimal for detecting and monitoring the abundance of membrane proteins and low-abundance proteins [21]. Therefore, the previous 2D-DIGE study may have failed to detect various salt-responsive changes in the abundance of *H. pylori* membrane proteins.

In the current study, we sought to identify *H. pylori* membrane proteins that change in abundance in response to alterations in environmental salt concentrations. We used both a label-free spectral counting approach, as well as Isobaric Tags for Relative Absolute Quantitation (iTRAQ). We selected the laboratory strain 26695 for analysis since it is a prototype strain with a detailed genome annotation, and we also analyzed strain 7.13, which can colonize Mongolian gerbils and promote the development of gastric cancer in this model [22]. We report on salt-responsive changes in protein abundance that were detected in both *H. pylori* strains, as well as strain-specific responses. We discuss how the observed changes in the bacteria in response to high salt conditions may influence interactions between *H. pylori* and gastric epithelial cells, as well as the host immune response to the bacteria, and propose that these changes may account, at least in part, for the increased risk of gastric cancer associated with a high salt diet.

Methods

Bacterial strains and growth conditions

H. pylori strain 26695 is a reference strain with a fully annotated genome sequence that was originally isolated from a patient that had gastritis [23, 24]. *H. pylori* strain 7.13 can colonize the stomach in a Mongolian gerbil model, sometimes resulting in gastric cancer [12, 13]. The complete genome sequence of a closely related strain (strain B8) was used in the current study when analyzing proteomic data [25]. Both strains have a functional *cag* T4SS. Strain 26695 lacks flagella and does not produce several OMPs (including HopZ, SabA, SabB, and HomB) [24]. Strain 7.13 does not produce VacA [25, 26]. For the proteomic studies, *H. pylori* strains were grown at 37°C in ambient air containing 5% CO₂ on either trypticase soy agar plates supplemented with 5% sheep blood, or in sulfite-free Brucella broth supplemented with 10% fetal bovine serum (BB-FBS) and containing varying concentrations of added sodium chloride: 0.25% (low salt), 0.5% (regular salt), or 1.1% (high salt) [15]. All proteomic analyses of the effect of salt concentration on protein abundance were performed by comparing bacteria grown in low salt conditions (0.25% added sodium chloride) with bacteria grown in high salt conditions (1.1% added sodium chloride). Bacteria were grown in the presence of 0.5% added sodium chloride for purposes of generating an unlabeled control preparation, as part of biotinylation experiments. The salt concentrations used in these experiments do not cause substantial alterations in bacterial growth [16].

Sample preparation for proteomic analysis

Bacteria were grown until late-log phase (absorbance 600 nm of approximately 0.7) as described previously [27]. Bacteria were pelleted at $3,500 \times g$ and resuspended in TNKCM (50 mM Tris, pH 7.4, 100 mM NaCl, 27 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂). Bacteria were pelleted again and resuspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail). Lysates were generated via sonication (4 pulses on ice at 15 sec each, with 45 sec rest between pulses). Unbroken cells were pelleted at $4,500 \times g$ for 10 min. Total membrane fractions were generated by ultracentrifugation of the lysates at $40,000 \times g$ for 30 min. Previous studies have shown that these total membrane fractions are enriched in inner and outer membrane proteins, but also contain detectable cytoplasmic and periplasmic proteins [27, 28]. Total membrane pellets were rinsed with lysis buffer and solubilized in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, protease inhibitor cocktail). Non-solubilized debris was removed by centrifugation at $21,000 \times g$ for 10 min. Total protein concentration was determined with BCA reagents (Pierce).

Biotinylation of surface-exposed proteins

Bacteria were grown to late-log phase in medium containing either 0.25% NaCl (low salt) or 1.1% NaCl (high salt), and surface-exposed proteins on intact bacteria were biotinylated and purified as described previously [27, 28]. Briefly, surface-exposed proteins were labeled using amine-reactive Sulfo-NHS-LC-biotin biotinylation reagent (Pierce). Bacteria were lysed by sonication and fractionated into a soluble protein fraction and a total membrane fraction, and then a preparation enriched in outer membrane proteins was prepared by

solubilizing the membrane fraction with 2% Zwittergent 3–14 (Fluka). Biotinylated proteins were selectively purified by using monomeric avidin magnetic beads (Bioclone) and were eluted by the addition of 5 mM D-Biotin (Sigma). Eluted proteins were precipitated with 20% trichloroacetic acid (TCA) (Sigma). To generate an unlabeled control preparation, bacteria were grown in medium containing 0.5% NaCl (normal salt), and the non-biotinylated bacteria were processed in parallel with the biotinylated bacteria. This biotinylation protocol allowed labeling of proteins on the surface of strain 26695, but similar experiments with strain 7.13 were less successful, possibly due to fragility of the latter strain during the multiple steps required for biotinylation.

Label-free analysis

For each strain analyzed, one culture was grown in high salt medium (1.1% NaCl) and one culture was grown in low salt medium (0.25% NaCl). Proteins in the membrane preparations were precipitated with ice-cold acetone overnight at -20°C . Following precipitation, samples were centrifuged at $18,000 \times g$ at 4°C , and precipitates were washed with cold acetone, dried, and reconstituted in 8M urea in 250 mM Triethyl ammonium bicarbonate (TEAB) (pH 8.0). Samples were reduced with Tris(2-carboxyethyl)phosphine (TCEP), alkylated with methyl methanethiosulfonate (MMTS), diluted with TEAB to obtain a solution containing 2M urea, and digested with sequencing-grade trypsin overnight at 37°C . For label-free LC-MS experiments, peptides were loaded onto a self-packed biphasic C18/SCX MudPIT column using a helium-pressurized cell (pressure bomb). The MudPIT column consisted of $360 \times 150 \mu\text{m}$ i.d. fused silica, which was fritted with a filter-end fitting (IDEX Health & Science) and packed with 6 cm of Luna SCX material ($5 \mu\text{m}$, $100\text{\AA}$) followed by 4 cm of Jupiter C18 material ($5 \mu\text{m}$, $300\text{\AA}$, Phenomenex). Once the sample was loaded, the MudPIT column was connected using an M-520 microfilter union (IDEX Health & Science) to an analytical column ($360 \mu\text{m} \times 100 \mu\text{m}$ i.d.), equipped with a laser-pulled emitter tip and packed with 20cm of C18 reverse phase material (Jupiter, $3\mu\text{m}$ beads, $300\text{\AA}$, Phenomenex). Using an Eksigent NanoLC HPLC and Autosampler, MudPIT analysis was performed with an 11-step salt pulse gradient (25, 50, 75, 100, 150, 200, 250, 300, 500, 750 mM, 1M ammonium acetate). Following each salt pulse, peptides were gradient-eluted from the reverse phase analytical column at a flow rate of 500 nL/min, and the mobile phase solvents consisted of 0.1% formic acid, 99.9% water (solvent A) and 0.1% formic acid, 99.9% acetonitrile (solvent B). For the peptides from the first 10 SCX fractions, the reverse phase gradient consisted of 2–40 %B in 90 min, followed by a 15 minute equilibration at 2 %B. For the last SCX-eluted peptide fractions, the peptides were eluted from the reverse phase analytical column using a gradient of 2–98 %B in 100 min, followed by a 10 min equilibration at 2 %B. Peptides were introduced via nano-electrospray into an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific), and the data were collected using a 17-scan event data-dependent method. Full scan (m/z 350–2000) spectra were acquired with the Orbitrap as the mass analyzer (resolution 60,000), and the sixteen most abundant ions in each MS scan were selected for collision-induced dissociation in the LTQ. An isolation width of 2 m/z , activation time of 10 ms, and 35% normalized collision energy were used to generate MS/MS spectra. The MSn AGC target value was set to 1×10^4 , and the maximum injection time was set to 100 ms. Dynamic exclusion was enabled, using a repeat count of 1 within 10 sec and exclusion duration of 30 sec. For peptide identifications, tandem mass

spectra were converted into DTA files using Scansifter and were searched using a custom version of Sequest (Thermo Fisher Scientific) operating on the Vanderbilt ACCRE computing cluster [29]. MS/MS spectra were searched against a concatenated forward and reverse (decoy) database containing the *H. pylori* 26695 or B8 strain subsets of UniprotKB protein database (www.uniprot.org). Additional search parameters included: trypsin enzyme specificity, monoisotopic masses used for searching product ions, and allowance of oxidation of methionine and MMTS modification of cysteine as variable modifications. Scaffold 4.1.1 (Proteome Software) was used to summarize and compare search results, where a minimum probability threshold of 95% was required for peptide identifications and data were filtered to a false-discovery rate (FDR) of <3% at the protein level.

To analyze preparations from biotinylated bacteria, shotgun proteomic analysis was performed by first resolving 50 µg of protein preparations approximately 1 cm using a 10% Novex precast gel, and then performing in-gel tryptic digestion at 37°C to recover peptides [30]. These peptides were analyzed via MudPIT essentially as described in [31, 32] and above, with the exception that data were acquired using an LTQ mass spectrometer (Thermo Scientific) and MudPIT separations performed using a NanoAquity HPLC system (Waters). Unlike the analysis of total membrane preparations, where data were collected over 11 salt steps, only 8 steps (~ 16 hr) were utilized for analyzing this less complicated cell surface proteome. Both the intact masses (MS) and 5 data-dependent fragmentation (MS/MS) spectra of the peptides were collected. The peptide MS/MS spectral data were searched against the *H. pylori* 26695 protein databases using Sequest [33]. Resulting identifications were collated and filtered using IDPicker 3 [34], where a minimum probability threshold of 95% was required for peptide identifications and data were filtered to a false-discovery rate (FDR) of <3% at the protein level. When analyzing data from biotinylated preparations, only spectra assigned to unique proteins were considered for spectral counting.

iTRAQ analysis

One 2-plex iTRAQ experiment was conducted for each *H. pylori* strain assayed. Total membrane preparations were generated as described above, and proteins were precipitated with ice-cold acetone overnight at -20°C. Following precipitation, samples were centrifuged at 18,000 × g at 4°C, and precipitates were washed with cold acetone, dried, and reconstituted in 8M urea in 250 mM TEAB (pH 8.0). Samples were reduced with 5 µL of 50 mM TCEP, alkylated with 2.5 µL of 200 mM MMTS, diluted 4-fold with TEAB to obtain a final solution containing 2M urea, and digested with sequencing-grade trypsin overnight. Peptides were then labeled with iTRAQ reagents according to the manufacturer's instructions (reporter tags: strain 26695, low salt = 114, high salt = 117; strain 7.13, low salt = 115, high salt = 117) (AB Sciex). For 50 µg of protein, 1 unit of labeling reagent was used. Labeling reagent was reconstituted in ethanol such that each protein sample was labeled at a final concentration of 90% ethanol, and labeling was performed for 2 hr. The resulting labeled peptides were then desalted by a modified Stage-tip method [35]. iTRAQ-labeled samples were mixed and acidified with TFA. A disc of C18 extraction membrane (Chrom Tech. Inc. C18 SPE Empore disk) was cored with a 16-gauge needle, and the cored piece of membrane was fitted tightly into a 200 µl pipet tip. Three milligrams of C18 resin (Phenomenex Jupiter C18, 5 µm particle size) was suspended in 200 µl of methanol and

loaded into the pipet tip containing the cored C18 membrane. The C18 material was packed into the tip using centrifugation to form a resin-packed C18 clean-up tip (resin tip). Multiple resin tips were used for desalting the mixed 2-plex iTRAQ samples. Resin tips were equilibrated with 0.1% TFA in HPLC-grade water, labeled peptides were loaded into the tip by centrifugation, washed with 0.1% TFA, and eluted with 100 μ l of 80% ACN containing 0.1% TFA. All eluted peptides were pooled together and dried by speed-vac centrifugation. Peptides were reconstituted in 0.1% formic acid, and peptides were loaded onto a self-packed biphasic C18/SCX MudPIT column using a helium-pressurized cell (pressure bomb). The MudPIT column consisted of $360 \times 150 \mu\text{m}$ i.d. fused silica, which was fritted with a filter-end fitting (IDEX Health & Science) and packed with 6 cm of Luna SCX material (5 μm , 100 $\text{\AA}$) followed by 4 cm of Jupiter C18 material (5 μm , 300 $\text{\AA}$, Phenomenex). Once the sample was loaded, the MudPIT column was connected using an M-520 microfilter union (IDEX Health & Science) to an analytical column (360 $\mu\text{m} \times 100 \mu\text{m}$ i.d.), equipped with a laser-pulled emitter tip and packed with 20 cm of C18 reverse phase material (Jupiter, 3 μm beads, 300 $\text{\AA}$, Phenomenex). Using an Eksigent NanoLC HPLC and Autosampler, MudPIT analysis was performed with a 13-step salt pulse gradient (0, 25, 50, 75, 100, 150, 200, 250, 300, 500, 750 mM, 1M, and 2M ammonium acetate). Following each salt pulse, peptides were gradient-eluted from the reverse analytical column at a flow rate of 500 nL/min, and the mobile phase solvents consisted of 0.1% formic acid, 99.9% water (solvent A) and 0.1% formic acid, 99.9% acetonitrile (solvent B). For the peptides from the first 11 SCX fractions, the reverse phase gradient consisted of 2–50 %B in 90 min, followed by a 15 min equilibration at 2 %B. For the last 2 SCX-eluted peptide fractions, the peptides were eluted from the reverse phase analytical column using a gradient of 2–98 %B in 100 min, followed by a 10 min equilibration at 2 %B. Peptides were introduced via nano-electrospray into a Q Exactive mass spectrometer (Thermo Scientific). The Q Exactive was operated in the data-dependent mode acquiring HCD MS/MS scans ($R = 17,500$) after each MS1 scan ($R = 70,000$) on the 18 most abundant ions using an MS1 ion target of 1×10^6 ions and an MS2 target of 5×10^4 ions. The maximum ion time for MS/MS scans was set to 100 ms, the HCD-normalized collision energy was set to 26, dynamic exclusion was set to 25 sec, and peptide match and isotope exclusion were enabled. Mass spectra were processed using the Spectrum Mill software package (version B.04.00) (Agilent Technologies). MS/MS spectra acquired on the same precursor m/z ($\pm 0.01 m/z$) within ± 1 sec in retention were merged. MS/MS spectra of poor quality which failed the quality filter by not having a sequence tag length >1 were excluded from searching. A minimum matched peak intensity requirement was set to 50%. For peptide identification, MS/MS spectra were searched against either the *H. pylori* strain 26695 or strain B8 subsets of the UniprotKB protein database (www.uniprot.org). Additional search parameters included: trypsin enzyme specificity with a maximum of three missed cleavages, ± 20 ppm precursor mass tolerance, ± 20 ppm (HCD) product mass tolerance, and fixed modifications including MMTS alkylation of cysteines and iTRAQ labeling of lysines and peptide N-termini. Oxidation of methionine was allowed as a variable modification. Autovalidation was performed such that peptide assignments to mass spectra were designated as valid following an automated procedure during which score thresholds were optimized separately for each precursor charge state and the maximum target-decoy-based false-discovery rate (FDR) was set to 1.0%. To obtain iTRAQ protein ratios, the median was calculated for all peptides assigned to each protein. Because of a high

interrelatedness among *H. pylori* outer membrane proteins (OMPs), only peptides that could be assigned to a single OMP were considered when calculating iTRAQ protein ratios.

Statistical analysis of assigned spectra

For label-free analysis of total membrane preparations, the identification of proteins enriched in one preparation compared to another was based on two criteria. First, the relative abundance of assigned spectra was calculated using a normalized ratio of spectral counts

(R_{sc}): $\log_2 \left(\frac{a+0.5}{b+0.5} \right) + \log_2 \left(\frac{b_{total}-b+0.5}{a_{total}-a+0.5} \right)$ [36]. For the identification of salt-responsive proteins, “a” indicates high salt and “b” indicates low salt. For the biotinylation experiment to identify biotinylated proteins, “a” indicates the biotinylated preparation and “b” indicates the unlabeled control. As a second criteria, the significance of differences in numbers of assigned spectra was calculated using a Fisher’s exact test (FET) with Benjamini-Hochberg (BH) multiple test correction. R_{sc} values were normalized to mean of zero prior to analysis. Proteins designated as salt-responsive had a normalized R_{sc} of < -0.585 or > 0.585 (corresponding to a 1.5 fold change), and a FET BH p value < 0.05 .

Triplicate samples of biotinylated preparations were analyzed and the resulting assigned spectra were merged prior to analysis [27]. Proteins in biotinylated samples were identified as enriched over the mock control preparation (no biotinylation) with a FET BH p value < 0.05 and $R_{sc} > 1$ [27]. Salt-responsive proteins were identified as described above for the label-free analysis.

To analyze iTRAQ data, frequency distribution histograms for $\log_2$ protein ratios were generated using Graphpad Prism 5.0. Ratios were binned to a value of 0.1. $\log_2$ ratios were then fitted to a normal distribution using least squares (non-linear) regression. The mean derived from the Gaussian fit was used to normalize $\log_2$ ratios for a bin center of zero. The mean and standard deviation derived from the Gaussian fit were used to generate a Z-score for each $\log_2$ ratio and calculate p values that were subsequently corrected for multiple comparisons by the BH method [37]. Proteins with a $p < 0.05$ were defined as significantly different in abundance. For strains 26695 and 7.13, p values of 0.05 correspond approximately to absolute $\log_2$ ratios of 1 and 0.8, respectively.

Non-linear regression analyses were performed in Graphpad Prism (Figure S1). Z-score, Fisher’s exact test, Student’s T-test, and multiple test corrections were calculated in R (<http://www.R-project.org>).

H. pylori motility assay

H. pylori motility was analyzed as described previously [38]. Briefly, overnight broth cultures of *H. pylori* 7.13 were back-diluted to an optical density at 600 nm of 0.1. 1- μ l aliquots of the bacterial suspensions were then stabbed into Brucella soft agar plates containing 5% FBS, 0.35% agar, and varying concentrations of sodium chloride. Diameters of bacterial halos were measured following a 5 day incubation of the plates at 37°C in a microaerobic atmosphere generated by GasPak EZ Campy packs (BD). At least 12 replicates were assayed for each condition.

Results

Salt-responsive proteins in strain 26695 identified by label-free and iTRAQ analyses

As an initial approach for identifying *H. pylori* proteins that differ in abundance depending on the sodium chloride concentration of the culture medium, we conducted experiments with the well-characterized *H. pylori* reference strain 26695. We cultured strain 26695 in either low-salt medium or high-salt medium, and generated subcellular fractions enriched in bacterial membrane proteins. We then analyzed the preparations by label-free MudPIT. Among 806 *H. pylori* proteins identified in the preparations, there was a significant difference in the number of assigned spectra for 66 proteins when comparing preparations from bacteria grown under low-salt and high-salt conditions (Table S1). Thirty-eight proteins were more abundant in preparations from bacteria grown in low salt conditions, and twenty-eight proteins were more abundant in preparations from bacteria grown in high salt conditions (Table S1).

As another approach to identify salt-responsive proteins, we used iTRAQ methods to analyze the membrane preparations from strain 26695, grown under high and low salt conditions, and identified 849 *H. pylori* proteins in the preparations. By using Z-score statistical analysis, we identified 22 of these proteins that were significantly different in abundance depending on the salt concentration (Table 1). Nine proteins were more abundant in bacteria grown under low salt conditions. These included the vacuolating toxin VacA, a VacA-like protein (FaaA), an outer membrane protein (FecA3) and multiple hypothetical proteins. Two proteins identified in this analysis (LysA and a hypothetical protein) are encoded by adjacent genes (HP0290 and HP0291) that are predicted to be members of an operon. Thirteen proteins were more abundant in bacteria grown under high salt conditions (Table 1). These included CagA, flagellar hook protein FlgE2 (HP0908), a fibronectin domain containing protein (HP0746), an amidase (AmiE), a sodium and chloride-dependent transporter (HP0497), a conserved hypothetical secreted protein (HP1464), and multiple other hypothetical proteins. A protein product of HP0964, previously annotated as a pseudogene, was also identified as more abundant in bacteria grown under high salt conditions (Table 1).

Eleven (50%) of the 22 proteins identified as salt responsive in the iTRAQ experiment were also identified as salt responsive in the label-free MudPIT experiment (Table 1 and Figure 1A). Six of the eleven proteins were more abundant in bacteria grown under high salt conditions --- CagA, AmiE, fibronectin-domain containing protein (HP0476), a product of the annotated pseudogene HP0964, and two hypothetical proteins (HP1464 and HP1588). Five of the eleven proteins were more abundant in bacteria grown under low salt conditions -- LysA (HP0290), FaaA, HP0664 (hypothetical protein), VacA, and FecA3.

As another approach to compare the results obtained by the label-free and iTRAQ methods, we analyzed the 66 salt-responsive proteins identified in the label-free experiments, and evaluated if the directionality of changes in protein abundance detected in the label-free experiments matched the directionality of changes in protein abundance detected in the iTRAQ experiments (Fig. S2A, S2B). In the label-free experiments, 28 proteins were upregulated under high salt conditions and 38 were upregulated under low salt conditions.

Similar salt-induced changes were observed in the iTRAQ experiments ($p < 0.001$, Student's T test, Fig. S2B). The same patterns were observed even when excluding the proteins that were identified by Z-score analysis of the iTRAQ data (Table 1, Figure S2B). These findings illustrate that the two different methods yielded similar patterns of results, and suggest that the changes in protein abundance were not limited to the relatively small set of salt-responsive proteins identified in the statistical analysis of the iTRAQ data.

Salt-responsive proteins in strain 7.13 identified by label-free and iTRAQ analyses

We next investigated the effects of changes in environmental salt concentration on protein abundance in *H. pylori* strain 7.13. In contrast to strain 26695, which lacks flagella and fails to colonize animal models, strain 7.13 produces flagella and can promote the development of gastric cancer in a Mongolian gerbil model [12, 13]. We cultured strain 7.13 in either low-salt medium or high-salt medium, prepared membrane fractions from the bacteria and analyzed the preparations by label-free MudPIT. A total of 1054 *H. pylori* proteins were identified in these preparations, and 222 of these proteins differed in abundance, depending on the salt concentration of the medium (Table S2). Among the 222 proteins, 111 were more abundant in bacteria grown under low salt conditions, and 111 other proteins were more abundant in bacteria grown under high salt conditions (Table S2). Next we analyzed membrane preparations of strain 7.13 grown under high and low salt conditions using iTRAQ. Among 1185 *H. pylori* proteins detected, we identified 56 proteins that were significantly different in abundance (Table 2). Nineteen proteins were more abundant in bacteria grown under low salt conditions (Table 2). These included two flagellar proteins (FlgE and FlaG), an iron (III) dicitrate transport protein (FecA3), and two subunits of quinone-reactive Ni-Fe hydrogenase. Thirty nine proteins were more abundant in bacteria grown under high salt conditions (Table 2). These included CagA, CagT, a metalloprotease (HP0506), and several outer membrane proteins (HopK, HopA, and HopD).

Among the 56 proteins produced by strain 7.13 that were identified as differentially abundant in iTRAQ experiments, 30 (54%) were identified as differentially abundant by both iTRAQ and label-free MudPIT (Table 2 and Figure 1B). Among these 30 proteins, 10 were enriched in bacteria grown under low salt conditions; these included the outer membrane protein FecA3 and flagellar hook protein FlgE3. The set of 20 proteins enriched in bacteria grown under high salt conditions included CagA, HopQ, CagT, HopD, HopA, and ComL.

We analyzed the 222 salt-responsive proteins from strain 7.13 that were identified in the label-free experiments, and evaluated if the directionality of changes in protein abundance detected in the label-free experiments matched the directionality of changes in protein abundance detected in the iTRAQ experiments (Figure S2A, S2C). In the label-free experiments, 111 proteins were upregulated under high salt conditions and 111 were upregulated under low salt conditions. Similar salt-induced changes were observed in the iTRAQ experiments ($p < 0.001$, Student's T test, Fig. S2C). The same patterns were observed even when excluding the proteins that were identified by Z-score analysis of the iTRAQ data (Table 2, Figure S2C). Thus, similar to the conclusions reached in studies of strain 26695, this analysis of the 7.13 data indicates that the two methodologic approaches yielded

similar patterns of results, and suggest that the changes in protein abundance were not limited to the relatively small set of salt-responsive proteins identified in the statistical analysis of the iTRAQ data.

Salt-responsive proteins identified in both strain 26695 and 7.13

The analysis of two different *H. pylori* strains in the experiments described above provided us with an opportunity to detect salt-responsive changes in protein abundance conserved in multiple strains. We identified 22 proteins that were salt-responsive in both strain 26695 and 7.13, based on a label-free spectral-counting approach (Table S3). By using iTRAQ, we identified 3 proteins that were salt-responsive in both strains (CagA, FecA3, and fibronectin domain protein) (Figure 1C and Table S3). Among the 22 proteins identified as salt-responsive in both 26695 and 7.13 based on label-free spectral counting experiments, 10 were identified as salt-responsive in at least one of the two iTRAQ analyses (Figure 1D). These included FaaA, FecA3, CagA, fibronectin-domain containing protein HP0746, HopQ, and HP0596 (corresponding to tumor necrosis factor alpha-inducing protein). Forty one proteins were identified as salt-responsive by at least one of the label-free analyses and also by at least one of the iTRAQ analyses (Figure 1C).

In addition to the salt-responsive alterations detected in both *H. pylori* strains, we also detected strain-specific responses to changes in salt concentrations (i.e., proteins that were identified as significantly different in abundance based on both label-free and iTRAQ methods for one strain, but not identified as salt-responsive by either method in the other strain) (Tables 1 and 2). For example, the outer membrane proteins HopA and HopD, flagellar protein FlaG, and the competence protein ComL were salt-responsive in strain 7.13 but not in strain 26695. In total we identified 13 proteins as salt-responsive in one strain but not the other (11 proteins from 7.13 and 2 proteins from 26695) (Tables 1 and 2). Additionally, we detected strain-specific differences in the directionality of salt-induced changes. AmiE was significantly more abundant in strain 26695 grown under high salt conditions (based on label-free spectral counting and iTRAQ), whereas AmiE was significantly more abundant in strain 7.13 grown under low salt conditions (based on label-free spectral counting). We also identified strain-specific differences in effects of salt on CheA and Omp8 (HopG), based on a label-free spectral counting approach (Table S3).

Changes in abundance of surface-exposed proteins in response to salt concentration

The label-free and iTRAQ experiments were performed by analyzing total membrane fractions, which contain both inner and outer membrane proteins. We hypothesized that, in comparison to analyses of total membrane fractions, low-abundance salt-responsive proteins present on the bacterial surface might be identified more efficiently by analyzing preparations enriched for surface-exposed proteins. Therefore, we grew *H. pylori* in either high salt or low salt concentrations as before, labeled surface-exposed proteins of intact bacteria with amine-reactive, cell-impermeable biotin, and purified the biotinylated proteins. We first analyzed the preparations of purified biotinylated proteins compared to mock preparations from control bacteria (not labeled with biotin and grown under normal salt conditions), using label-free MudPIT. Among 639 *H. pylori* proteins detected in these preparations, we identified 146 as putative surface-exposed proteins (Table S4), many of

which were also identified by the same approach in a previous study [27]. We next compared the biotinylated preparations from bacteria grown in high salt conditions with biotinylated preparations from bacteria grown in low salt conditions, and identified 21 of the 146 putative surface-exposed proteins as differentially abundant (Table 3). Most of these (15 of 21) were more abundant in the preparations from bacteria grown under low salt conditions than in the preparations from bacteria grown under high salt conditions. These included VacA-like proteins ImaA (HP0289) and FaaA (HP0609/10), BamA, ComH, FecA3, several annotated OMPs (AlpB, HorB, and HP0358), two conserved hypothetical secreted proteins (HP0235 and HP1117), and several hypothetical proteins (Table 3). Proteins identified as enriched in the high salt preparations compared to low salt preparations included HP1124 (a hypothetical protein that may be an outer membrane channel, based on BLAST conserved domain search), and fibronectin type 3 domain protein (HP0746) (Table 3).

Three proteins produced by strain 26695 (FecA3, fibronectin-domain protein HP0746, and FaaA) were identified as salt-responsive based on all three of the experimental approaches (label-free MudPIT, iTRAQ, and biotinylation) (Fig. 1E), and 14 proteins were identified as salt-responsive by two of the three methods (Fig. 1E). In addition to the proteins listed previously (detected by both label-free methods and iTRAQ), these included FaaA (HP0609) and three hypothetical proteins (HP0358, HP1527, and HP1124).

Salt-dependent changes in bacterial motility

Label-free and iTRAQ analysis of strain 7.13 revealed that the abundance of multiple proteins associated with flagellar functions was reduced when the bacteria were cultured in high salt medium. We hypothesized that these shifts in protein abundance would have an effect on bacterial motility. To test this hypothesis, we analyzed the motility of *H. pylori* 7.13 in soft agar containing a range of sodium chloride concentrations. We observed a significant decrease in bacterial motility in response to high salt conditions, and the effect of salt concentration on motility was dose-dependent (Figure 2).

Discussion

In this study, we used both label-free and iTRAQ methods to identify *H. pylori* membrane proteins that change in abundance in response to alterations in environmental salt concentrations. In addition, we analyzed the responses of two different *H. pylori* strains to alterations in salt concentrations. These methods allowed us to identify salt-responsive proteins in a given strain by more than one proteomic approach, as well as proteins that were salt-responsive in more than one *H. pylori* strain.

When comparing the iTRAQ results to the label-free results for a particular strain, we were able to confirm approximately 50% of the iTRAQ results by label-free methods (Figure 1). Conversely, a lower proportion of the label-free results was confirmed by iTRAQ results. This pattern is attributable to the use of different criteria for identifying salt-responsive proteins in the iTRAQ experiments compared to the label-free experiments. The differences in iTRAQ and label-free results also reflect distinct strengths and limitations of the two approaches. For example, label-free spectral counting is optimal for detecting differences in

abundance of proteins with many assigned spectra, whereas iTRAQ is able to quantify differences in the abundance of proteins with fewer assigned spectra.

In a previous study of strains 26695 and 7.13, 31 salt-responsive *H. pylori* proteins were identified by using 2D-DIGE [15]. Several salt-responsive proteins identified in the previous 2D-DIGE analysis (for example, CagA, HopQ, FaaA, and HP1588) were also identified by multiple methods in the current study. Several other proteins designated as salt-responsive in the previous 2D-DIGE analysis (HP0014, HP0221, HP0289, HP0377, HP0630, and HP0680) underwent similar salt-dependent changes in abundance in the current experiments, but the changes were not deemed to be statistically significant. Multiple factors account for the relatively small numbers of salt-responsive proteins identified in both the previous 2D-DIGE study and the current study. For example, the current study focused on membrane-associated proteins, whereas the 2D-DIGE was performed with whole cell lysate. Another important consideration is that 2D-DIGE is suboptimal for analysis of membrane proteins and low-abundance proteins. The methods utilized in this study permitted an analysis of such proteins with a higher level of sensitivity than was possible using 2D-DIGE. Thus, we were able to identify several salt-responsive proteins that were not previously demonstrated. For example, fibronectin domain-containing protein HP0746 and the outer membrane protein FecA3 were shown to be salt-responsive in the current study by multiple methods and in two different *H. pylori* strains. Currently very little is known about the function of HP0746. BLAST analysis indicates that this protein contains multiple fibronectin type 3 domains, suggesting that HP0746 may function as an adhesin or an extracellular glycohydrolase [39, 40]. FecA3 is one of several annotated outer membrane iron dicitrate transporter proteins. The proteomic data indicate that both *H. pylori* strains analyzed in this study produce three distinct FecA proteins (FecA1, FecA2, and FecA3). FecA1 and FecA2 have been previously reported to be regulated by Fur [41, 42] and the current results indicate that FecA3 is regulated by environmental salt concentrations.

A comparative analysis of strains 26695 and 7.13 identified numerous proteins that were salt-responsive in both strains. Recapitulation of the findings in multiple strain backgrounds provides added confidence in the designation of these proteins as salt-responsive. We also observed 14 examples of strain-specific responses (proteins that were identified as salt responsive in one strain based on both label-free and iTRAQ methods, but not identified as salt responsive in the other strain by either proteomic method). For example, HopA, HopD, FlaG, and ComL were salt-responsive in strain 7.13 but not in strain 26695. Strikingly, salt had opposite effects on AmiE in the two strains (more abundant in high salt conditions for strain 26695 and more abundant in low salt conditions for strain 7.13). These strain-specific differences might be attributable to the high level of genetic diversity that exists among *H. pylori* strains. For example, there might be variations in nucleotide sequence motifs recognized by salt-responsive regulators, or there might be variations in the regulatory systems that mediate salt-responsive changes. The observed strain-specific variation in results is consistent with previous reports of strain-specific responses to environmental stimuli in *H. pylori* [43].

Previous studies have shown that transcriptional alterations account for the changes in abundance of several *H. pylori* proteins in response to altered salt concentration [15, 16, 43].

However, it seems possible that some of the observed changes may also be mediated by post-transcriptional changes (including changes in transcript stability or changes in protein stability). Analysis of promoter regions and untranslated regions of genes encoding the salt-responsive proteins identified in this study did not readily identify any conserved sequence motifs that might be mechanistically involved in the observed salt-responsive changes. Further studies will be required to elucidate the molecular mechanisms underlying the observed salt-induced changes in abundance of individual proteins.

Our analysis of strain 7.13 revealed that there was a lower abundance of multiple proteins associated with flagellar activity when the bacteria were grown in high salt conditions. Most of these changes were not detected with strain 26695, which does not produce flagella; flagellar hook protein FlgE2 (HP0908) was the only salt responsive protein associated with flagellar activity detected in the iTRAQ analysis of strain 26695. Concordant with the proteomic data for strain 7.13, we detected decreased bacterial motility of this strain under high salt conditions. Previous studies showed that the salt concentrations employed in these experiments have minimal effects on the growth rate of *H. pylori* [16], but it is possible that slightly impaired growth at high salt concentrations may contribute to the reductions in halo size observed in the soft agar assay. Changes in *H. pylori* motility in response to altered salt concentration could have important consequences for the bacteria. For example, if the bacteria are less motile, this may impair their ability to move within the mucus layer in response to pH or nutrient availability, which could potentially lead to alterations in bacterial load. Studies in mouse models have shown that there is typically a reciprocal relationship between *H. pylori* density and severity of the gastric inflammatory response [44], and therefore, alterations in the bacterial load may lead to changes in the gastric mucosal inflammatory response. In addition, decreased motility may facilitate increased bacterial adhesion to the host tissue. Similar to the results of the current study, decreased bacterial motility in response to high salt concentrations has also been observed in studies of other bacterial species [45].

Among the salt-responsive proteins identified in this study, several have previously been shown to have important roles in *H. pylori* colonization of the stomach. For example, FecA3 is required for *H. pylori* colonization of the mouse stomach [42], and a *faaA* mutant was outcompeted by a wild type strain [38]. The impaired colonization of the *faaA* mutant may be attributable to flagellar abnormalities and a motility defect [38]. The functions of most of the other salt-responsive proteins *in vivo* have not yet been studied in any detail.

In summary, these results provide new insight into the effect of environmental salt concentration on the *H. pylori* membrane proteome. In future studies, it will be important to analyze further the functional consequences of the observed changes in protein abundance. In addition to the observed changes in motility that occur in response to altered salt concentrations, we speculate that altered abundance of certain salt-responsive proteins may enhance the ability of *H. pylori* to survive salt stress. We further speculate that the changes in abundance of surface-exposed proteins that occur following exposure of *H. pylori* to high salt concentrations may lead to altered interactions of the bacteria with gastric epithelial cells and immune cells (for example, changes in bacterial adherence and changes in production of cytokines), which could influence the severity of the gastric inflammatory

response. In future studies, it will also be important to analyze *H. pylori* strains isolated from patients who chronically consume a high salt diet and determine if these strains exhibit differences in proteomic profiles compared to isolates from patients who consume low salt diets. Ultimately, studies of *H. pylori* responses to altered salt concentrations may lead to a better understanding of the mechanisms by which a high salt diet contributes to an increased risk of gastric adenocarcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Clinical Relevance

Approximately half of the world's population is colonized by *Helicobacter pylori*. Colonization of the stomach with *H. pylori* is a risk factor for the development of gastric cancer, currently the third leading cause of cancer-related death. A high salt diet has also been associated with the development of gastric cancer. Previous studies have shown that the cultivation of *H. pylori* in high-salt conditions leads to increased production of CagA, a bacterial protein that enters host cells and causes numerous alterations. In this study, we identify numerous additional *H. pylori* proteins that change in abundance in response to the salt concentration of the bacteriologic culture medium. We propose that the increased risk of gastric cancer associated with a high salt diet is attributable in part to salt-induced changes in the *H. pylori* proteome.

Fig. 1. Identification of salt-responsive proteins by multiple methods

Venn diagrams showing: A) salt-responsive proteins in strain 26695 detected by label free methods compared to iTRAQ methods, B) salt-responsive proteins in strain 7.13 detected by label-free methods compared to iTRAQ methods, C) the numbers of salt-responsive proteins identified by iTRAQ analysis of the indicated strains. “26695 and/or 7.13 label-free” indicates salt-responsive proteins identified by label-free analysis of at least one strain. D) Salt-responsive proteins identified by label-free analysis of the indicated strains. “26695 and/or 7.13 iTRAQ” indicates salt-responsive proteins identified by iTRAQ analysis of at least one strain. E) Salt-responsive proteins in strain 26695 identified by the three indicated methods.

Fig. 2. Effect of salt concentration on *H. pylori* motility

H. pylori strain 7.13 was stabbed into Brucella soft agar plates containing 5% FBS, 0.35% agar, and the indicated concentrations of added sodium chloride. Diameters of bacterial halos were measured after incubation for 5 days. The indicated p values were calculated by Student's T test.

Table 1

Salt-responsive proteins in strain 26695 identified by iTRAQ analysis

Proteins more abundant in low salt compared to high salt				
Accession	Uniprot	KEGG	Fold Difference	Description
HP1400 ^{##}	O25950	Transport	2.53	iron(III) dicitrate transport protein (FecA3)
HP0664 ^{*&}	K4NED0	NA	2.36	hypothetical protein
HP0887 [*]	P55981	Pathogenesis	2.36	vacuolating cytotoxin
HP0802	O08315	riboflavin biosynthetic process	2.33	GTP cyclohydrolase II (RibA)
HP0290 [*]	P56129	lysine biosynthetic process	2.30	diaminopimelate decarboxylase (dap decarboxylase) (LysA)
HP1532	O26060	glutamine metabolic process	2.27	glucosamine fructose-6-phosphate aminotransferase (isomerizing) (GlmS)
HP0721	O25423	NA	2.08	hypothetical protein
HP0291	O25064	chorismate metabolic process	2.02	hypothetical protein
HP0610 ^{##}	O25331	NA	2.01	toxin-like outer membrane protein (FaaA)

Proteins more abundant in high salt compared to low salt				
Accession	Uniprot	KEGG	Fold Difference	Description
HP0964 [*]	O25617	NA	3.86	hypothetical protein
HP0574	O25298	pentose-phosphate shunt	3.38	ribose 5-phosphate isomerase B (RpiB)
HP0547 [*]	P55980	toxin transport	3.16	cag pathogenicity island protein (CagA)
HP1584	P55996	proteolysis	3.15	sialoglycoprotease (Gcp)
HP1248	P56123	nucleic acid phosphodiester bond hydrolysis	2.92	virulence associated protein homolog (VacB)
HP0294 [*]	O25067	nitrogen compound metabolic process	2.76	aliphatic amidase (AmiE)
HP1464 [*]	O26000	NA	2.58	conserved hypothetical secreted protein
HP0908 ^l	O25566	NA	2.37	flagellar hook (FlgE2)
HP0497	O25239	transmembrane transport	2.37	sodium- and chloride-dependent transporter
HP1588 ^{*&}	O26107	NA	2.32	conserved hypothetical protein
HP0337	O25104	NA	2.23	hypothetical protein
HP1124	O25749	NA	2.13	hypothetical protein

Proteins more abundant in high salt compared to low salt				
Accession	Uniprot	KEGG	Fold Difference	Description
HP0746 ^{*,#}	O25442	NA	1.97	fibronectin domain-containing protein

* Also significant in label-free analysis

Also significant in biotinylation experiments

& Significant in iTRAQ and label-free analyses of strain 26695, but not significant in either analysis of strain 7.13

! Strain 26695 produces two distinct FlgE proteins, FlgE1 (HP0870) and FlgE2 (HP0908).

NA: not assigned

Table 2

Salt-responsive proteins in strain 7.13 identified by iTRAQ analysis

Proteins more abundant in low salt compared to high salt					26695 Homolog Locus Tag
Accession	Uniprot	KEGG	Fold difference	Description	
HPB8_1702*	D7FGF2	Transport	2.45	Putative iron(III) dicitrate transport protein (FecA3)	HP1400
HPB8_1645	D7FG95	metabolic process	2.43	Membrane-bound lytic murein transglycosylase D	HP1572
HPB8_831	D7FDY1	oxidation-reduction process	2.32	Quinone-reactive Ni/Fe hydrogenase, small subunit (HydA)	HP0631
HPB8_1116	D7FER6	NA	2.26	Putative uncharacterized protein	HP0467
HPB8_258*&	D7FCA8	heme binding	2.18	Cytochrome c553	HP1227
HPB8_625	D7FDC5	cellular aromatic compound metabolic process	2.15	4-oxalocrotonate tautomerase (DmpI)	HP0924
HPB8_829*	D7FDX9	NA	2.1	Modulator of drug activity B (MDA66)	HP0630
HPB8_958*&	D7FEA8	NA	2.07	Flagellar protein FlaG	HP0751
HPB8_844*	D7FDZ4	oxidation-reduction process	2.05	NAD(P)H-flavin oxidoreductase	HP0642
HPB8_822	D7FDX2	cell wall organization	2.02	UDP-N-acetylmutarimide-L-alanine ligase	HP0623
HPB8_188	D7FC38	NA	2.00	Putative uncharacterized protein	NA
HPB8_832*&	D7FDY2	oxidation-reduction process	1.98	Quinone-reactive Ni/Fe hydrogenase, large subunit (HydB)	HP0632
HPB8_176*&	D7FC26	translation	1.87	50S ribosomal protein L18	HP1303
HPB8_153	D7FC03	NA	1.83	Putative uncharacterized protein	HP1326
HPB8_610	D7FDB0	NA	1.83	Putative uncharacterized protein	HP0938
HPB8_1264*&	D7FF65	transmembrane transport	1.8	Dipeptide ABC transporter, periplasmic dipeptide-binding protein (DppA)	HP0298
HPB8_195	D7FC45	NA	1.78	Putative uncharacterized protein	HP1286
HPB8_1079*!	D7FEM9	bacterial-type flagellum-dependent cell motility	1.77	Flagellar hook protein FlgE3	HP0870
HPB8_2*	D7FBK2	NA	1.76	Putative uncharacterized protein	HP1527

Proteins more abundant in high salt compared to low salt					26695 Homolog Locus Tag
Accession	Uniprot	KEGG	Fold difference	Description	
HPB8_475*&	D7FCX5#	NA	3.47	Putative uncharacterized protein	HP0423HP1412

Proteins more abundant in high salt compared to low salt						26695 Homolog Locus Tag	
Accession	Uniprot	KEGG	Fold difference	Description			
HPB8_1569	D7FG19#	NA	3.47	Putative uncharacterized protein		NA	
HPB8_1195*	D7FEZ5	NA	3.07	Putative uncharacterized protein		HP1076	
HPB8_524	D7FD24#	NA	2.56	Putative uncharacterized protein		NA	
HPB8_555	D7FD55#	NA	2.56	Putative uncharacterized protein		HP1405	
HPB8_1336*&	D7FFD6	NA	2.53	Outer membrane protein HopA		HP0229	
HPB8_1699	D7FGE9	NA	2.53	Putative uncharacterized protein		HP1397	
HPB8_957*&	D7FEA7	NA	2.48	Putative uncharacterized protein		HP0750	
HPB8_1043	D7FEJ3	NA	2.43	Putative uncharacterized protein		HP0833	
HPB8_385*	D7FCN5	NA	2.4	cysteine-rich protein X HcpX		HP1117	
HPB8_513*	D7FD13	NA	2.23	Putative uncharacterized protein		NA	
HPB8_877*	D7FE27	NA	2.21	Putative uncharacterized protein		HP0674	
HPB8_p0002	D7GAX3	NA	2.2	Putative uncharacterized protein		NA	
HPB8_641*	D7FDE1	DNA duplex unwinding	2.17	Rep helicase, single-stranded DNA-dependent ATPase (Rep)		HP0911	
HPB8_1582	D7FC32	polysaccharide metabolic process	2.17	Mannose-1-phosphate guanylyltransferase		HP0043	
HPB8_622	D7FDC2	proteolysis	2.16	Protease HcpX homolog		HP0927	
HPB8_1057*	D7FEK7	DNA modification	2.15	Type I restriction enzyme, S subunit		HP0790	
HPB8_1600*&	D7FG51	NA	2.12	Outer membrane protein HopD		HP0025	
HPB8_872	D7FE22	metabolic process	2.04	NAD-dependent deacetylase		NA	
HPB8_28	D7FBM8	NA	1.99	Outer membrane protein HorK		HP1501	
HPB8_711*	D7FDL1	NA	1.98	Cag pathogenicity island protein T		HP0532	
HPB8_690	D7FDJ0	metabolic process	1.97	Uncharacterized metalloprotease yebA		HP0506	
HPB8_89	D7FBT9	translation	1.95	50S ribosomal protein L34		HP1447	
HPB8_741*	D7FDP1	toxin transport	1.92	Cytotoxin-associated protein A		HP0547	
HPB8_998	D7FEE8	DNA modification	1.89	Type I restriction enzyme, S subunit		HP0790	
HPB8_1204	D7FF04	NA	1.87	Putative uncharacterized protein		HP1085	
HPB8_794*	D7FDU4	NA	1.84	Putative uncharacterized protein		HP0596	
HPB8_1567*&	D7FG17	NA	1.82	Putative uncharacterized protein		HP1411 HP0424	

Proteins more abundant in high salt compared to low salt					
Accession	Uniprot	KEGG	Fold difference	Description	26695 Homolog Locus Tag
HPB8_865	D7FE15	mRNA processing	1.8	Ribonuclease 3	HP0662
HPB8_537	D7FD37	DNA topological change	1.79	DNA topoisomerase I	HP0440
HPB8_897	D7FE47	metabolic process	1.79	Acetone carboxylase, alpha subunit	HP0696
HPB8_953*	D7FEA3	NA	1.79	Putative uncharacterized protein	HP0746
HPB8_1547*&	D7FFZ7	NA	1.79	Putative uncharacterized protein comL	HP1378
HPB8_319*	D7FCG9	NA	1.78	Outer membrane protein HopQ	HP1177
HPB8_729	D7FDN0	NA	1.78	Putative uncharacterized protein	HP0513
HPB8_1047*	D7FEJ7	NA	1.78	Putative uncharacterized protein	HP0836/HP0837
HPB8_972*	D7FEC2	NA	1.77	Putative uncharacterized protein	HP0764
HPB8_24	D7FBM4	oxidation-reduction process	1.75	Riboflavin biosynthesis protein (RibG)	HP1505
HPB8_375*	D7FCM5	cell outer membrane	1.75	Peptidoglycan-associated lipoprotein	HP1125

* Also significant in label-free analysis

& Significant in iTRAQ and label-free analyses of strain 7.13, but not significant in either analysis of strain 26695

! Strain 7.13 produces two distinct FlgE proteins, FlgE1 and FlgE3.

NA: not assigned

Table 3
Salt-responsive proteins in strain 26695 identified by analysis of biotinylated intact bacteria

Proteins more abundant in low salt compared to high salt									
Accession	Assigned Spectra			Fold difference			Uniprot	KEGG	Description
	Biotinylated High Salt	Biotinylated Low Salt	Unlabeled Normal Salt Control	Low vs. High	High vs. Control	Low vs. Control			
HP0289	3	20	0	6.58	2.16	13.33	O25063	Protein Binding	toxin-like outer membrane protein (ImaA)
HP0235	8	25	0	3.37	5.25	16.57	O25021	metabolic process	conserved hypothetical secreted protein
HP1117	55	131	5	2.67	3.09	7.7	O25742	NA	conserved hypothetical secreted protein
HP0609*	62	141	18	2.55	1.03	2.46	O25330	protein binding	toxin-like outer membrane protein (FaaA)
HP0913	142	291	25	2.31	1.69	3.63	O25571	NA	outer membrane protein (omp21)
HP0610**	153	307	30	2.26	1.52	3.2	O25331	NA	toxin-like outer membrane protein (FaaA)
HP0645*	55	97	2	1.98	6.82	12.6	O25362	NA	soluble lytic murein transglycosylase (Slt)
HP1400**	140	236	9	1.9	4.49	7.95	O25950	transport	iron(III) dicitrate transport protein (FecA3)
HP0358*	155	260	4	1.89	10.47	18.45	O25125	NA	hypothetical protein
HP1527*	192	314	7	1.84	7.74	13.31	O26055	NA	ComH
HP1333	51	83	1	1.82	10.55	18.01	O25891	NA	hypothetical protein
HP0655	163	256	12	1.77	3.96	6.55	O25369	protein insertion into membrane	BamA
HP0252	82	123	1	1.68	16.85	26.55	O25034	NA	outer membrane protein (omp7)
HP1173	163	233	4	1.61	11.04	16.59	O25787	NA	hypothetical protein

Proteins more abundant in low salt compared to high salt						
Accession	Assigned Spectra			Fold difference		
	Biotinylated High Salt	Biotinylated Low Salt	Unlabeled Normal Salt Control	Low vs. High	High vs. Control	Low vs. Control
HP0127	161	218	15	1.52	3.17	4.51
						outer membrane protein (omp4)

Proteins more abundant in high salt compared to low salt						
Accession	Assigned Spectra			Fold difference		
	Biotinylated High Salt	Biotinylated Low Salt	Unlabeled Normal Salt Control	Low vs. High	High vs. Control	Low vs. Control
HP1376	16	2	0	5.88	10.22	1.63
HP1187	36	13	0	2.41	22.59	8.79
HP1561	68	32	3	1.88	6.05	3.02
HP1124 [#]	608	326	10	1.67	17.68	9.99
HP0746 ^{*#}	238	140	1	1.52	48.88	30.31
HP0103 [*]	91	53	1	1.52	18.83	11.59
						lipid A biosynthetic process
						(3R)-hydroxymyristoyl-acyl carrier protein dehydratase (FabZ)
						hypothetical protein
						iron(III) ABC transporter, periplasmic iron-binding protein (CeutE)
						hypothetical protein
						fibronectin type 3 domain protein
						methyl-accepting chemotaxis protein (tlpB)

* Also significant in label-free analysis of 26695 total membrane preparations

[#] Also significant in 26695 iTRAQ experiment