Helicobacter pylori Adaptation In Vivo in Response to a High-Salt Diet

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Helicobacter pylori exhibits a high level of intraspecies genetic diversity. In this study, we investigated whether the diversification of H. pylori is influenced by the composition of the diet. Specifically, we investigated the effect of a high-salt diet (a known risk factor for gastric adenocarcinoma) on H. pylori diversification within a host. We analyzed H. pylori strains isolated from Mongolian gerbils fed either a high-salt diet or a regular diet for 4 months by proteomic and whole-genome sequencing methods. Compared to the input strain and output strains from animals fed a regular diet, the output strains from animals fed a high-salt diet produced higher levels of proteins involved in iron acquisition and oxidative-stress resistance. Several of these changes were attributable to a nonsynonymous mutation in fur (fur-R88H). Further experiments indicated that this mutation conferred increased resistance to high-salt conditions and oxidative stress. We propose a model in which a high-salt diet leads to high levels of gastric inflammation and associated oxidative stress in H. pylori-infected animals and that these conditions, along with the high intraluminal concentrations of sodium chloride, lead to selection of H. pylori strains that are most fit for growth in this environment.

H. pylori is a Gram-negative bacterium that persistently colonizes the human stomach in half of the world’s population (1, 2). H. pylori exhibits a high level of intraspecies genetic diversity, characterized by marked variation among strains in gene content, as well as a high level of variation in the nucleotide sequences of individual genes (3, 4). A high rate of allelic diversity is attributable to both a high mutation rate and intraspecies recombination (5, 6).

Most H. pylori-infected persons remain asymptomatic, but the presence of this bacterium increases the risk of peptic ulcer disease and gastric cancer (1, 2, 7, 8). The clinical outcome of H. pylori infection is determined by a combination of bacterial, host, and environmental factors. For example, H. pylori strains containing the cag pathogenicity island (PAI) are associated with a higher risk of symptomatic disease than are strains that lack the cag PAI (9). One of the proteins encoded by the cag PAI, CagA, enters host cells and causes numerous cellular alterations linked to malignant transformation (10). The cag PAI also encodes multiple protein components of a type IV secretion system required for entry of CagA into host cells (11). H. pylori strains that secrete specific types of the VacA toxin are also linked to adverse disease outcome, in comparison to strains that secrete relatively nontoxic forms of the VacA protein (12, 13).

One of the environmental factors associated with increased gastric cancer risk is a high-salt diet (14). Epidemiologic studies have demonstrated a link between high dietary salt consumption and increased gastric cancer risk in many parts of the world (15–17), and a high-salt diet also increases the gastric cancer risk in animal models (18–21). For instance, in H. pylori-infected Mongolian gerbils treated with a chemical carcinogen, high dietary salt consumption was associated with an increased incidence of gastric cancer (21). Another study reported that H. pylori infection and a high-salt diet could independently induce atrophic gastritis and intestinal metaplasia in Mongolian gerbils (18). More recently, administration of a high-salt diet to Mongolian gerbils infected with an H. pylori strain harboring a functionally active cag PAI-encoded type IV secretion system was shown to increase the incidence of gastric cancer, compared to what was observed in infected animals fed a regular diet (22). Infected animals fed a high-salt diet had more severe gastric inflammation, a higher gastric pH, greater parietal cell loss, greater gastric expression of interleukin-1β, and lower gastric expression of hepcidin and hydrogen potassium ATPase than those on a regular diet (22). Several studies have demonstrated that H. pylori responds in vitro to alterations in the sodium chloride concentration of the culture medium. Salt-responsive changes include alterations in bacterial morphology, altered expression of specific H. pylori genes, and altered abundance of specific H. pylori proteins (23–26). Increased production of CagA in response to high-salt conditions may be one factor that accounts for the increased risk of gastric cancer associated with a high-salt diet (22, 24, 25).

In the present study, we tested the hypothesis that prolonged exposure of H. pylori in vivo to the gastric environmental conditions associated with a high-salt diet might lead to the emergence of strains adapted to these conditions. We show that the production of proteins involved in iron acquisition and oxidative-stress...
greater resistance in \textit{H. pylori} strains cultured from animals on a high-salt diet differs from that of the input strain and strains isolated from animals on a regular diet. We show that a nonsynonymous mutation in \textit{fur} (encoding the Fur transcriptional regulator) accounts for the altered production of several of the differentially abundant proteins, and we show that strains harboring this \textit{fur} mutation exhibit increased resistance to high-salt conditions and oxidative stress \textit{in vitro}. These results reveal that the evolution of \textit{H. pylori} within a host can be shaped by the composition of the diet. Since consumption of a high-salt diet in a setting of \textit{H. pylori} infection results in gastric environmental conditions that are markedly different from those associated with a regular-salt diet (22), we propose that there is strong positive selection for \textit{H. pylori} strains that are most fit for growth in the high-salt gastric environment.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains and culture methods.} \textit{H. pylori} strain B128 was originally isolated from the stomach of a human with gastric ulcer disease and was subsequently used for orogastric infection of Mongolian gerbils (27, 28). The strain isolated from one of these gerbils (B128 7.13) was designated strain 7.13 in a previous study (27) (Fig. 1). We recently conducted a study in which we infected a cohort of gerbils with strain 7.13 and fed the infected animals either a regular or a high-salt diet. Output strains from nine gerbils fed a regular diet (RSO) and nine gerbils fed a high-salt diet (HSO) were subsequently analyzed. Strains from two gerbils on a regular diet (RSO241 and RSO242) and two gerbils on a high-salt diet (HSO1 and HSO123) and input 7.13 strain were analyzed by a proteomic approach. Strains RSO241 and HSO1 and the input strain (four single colonies of each) were analyzed by whole-genome sequencing.

\textbf{Proteomic analyses.\textit{}} Two independent proteomic experiments were performed. Experiment 1 compared the proteome of strain 7.13, which was used previously for infection of gerbils (22) (input strain), with that of output strains RSO242 and HSO123, and experiment 2 compared the proteome of the input strain with that of output strains RSO241 and HSO1. \textit{H. pylori} strains were grown overnight in BB-FBS to an optical density of 0.5. Bacteria were harvested by centrifugation at 3,500 \textit{g}, and the pellets were resuspended in phosphate-buffered saline. Following a second centrifugation at 3,500 \textit{g}, pellets were resuspended in Tris-buffered saline. Peptide mass spectra were acquired using a QSTAR LQ clinical and were analyzed using SEQUEST (full tryptic specificity). Bacteria were lysed by sonication, and lysates were clarified by centrifugation. These preparations were then analyzed by multidimensional protein identification technology (MudPIT) as described previously (29–31). Peptide tandem mass spectra were queried with SEQUEST (full tryptic specificity) against an \textit{H. pylori} strain B8 (a closely related strain for which a complete genome sequence is available) (32) database to which both common contaminants and reversed versions of the proteins had been appended. B8 is the designation given previously to a strain that was cultured from Mongolian gerbils infected with \textit{H. pylori} strain B128 (32).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Schematic illustrating genomic and proteomic analyses of \textit{H. pylori} strains. Gerbils were infected with \textit{H. pylori} strain 7.13, and the animals were fed either a regular or a high-salt diet. Output strains from nine gerbils fed a regular diet (RSO) and nine gerbils fed a high-salt diet (HSO) were subsequently analyzed. Strains from two gerbils on a regular diet (RSO241 and RSO242) and two gerbils on a high-salt diet (HSO1 and HSO123) and input 7.13 strain were analyzed by a proteomic approach. Strains RSO241 and HSO1 and the input strain (four single colonies of each) were analyzed by whole-genome sequencing.}
\end{figure}
and B8 each originated from the same parent strain (human isolate B128) and each was generated by experimentally infecting gerbils with strain B128, these lineages are predicted to be very closely related. Resulting clones from the same parent strain (human isolate B128) and each was generated by experimentally infecting gerbils with strain B128, therefore, these lineages are predicted to be very closely related.

**Real-time PCR.** Overnight broth cultures of *H. pylori* output strains were subcultured into fresh brucella broth and grown for 5 h (OD600 of ~0.5 to 0.6). Total RNA was isolated from *H. pylori* with TRIzol reagent (Gibco). RNA samples were digested with RQ1 RNase-free DNase (Promega) to remove contaminating DNA, and the RNA samples were then subjected to a cleanup step with RNaseasy columns (Qiagen). cDNA synthesis was performed with 100 ng of purified RNA with the first-strand iScript cDNA synthesis kit (Bio-Rad). As controls, first-strand cDNA synthesis reactions without reverse transcriptase were carried out in parallel. The cDNA and control preparations were digested 1:20 and used in real-time PCRs. Real-time PCR was performed with an ABI Real Time PCR machine (Step One Plus) with SYBR green as the fluorochrome. Transcript abundance was assessed by the ΔΔCt method (Ct, cycle threshold), with each transcript normalized to the abundance of the 16S rRNA internal control. The normalized transcript signal for each biological sample was then divided by similarly normalized values from control samples to obtain a relative expression ratio. The primers used for real-time analysis were as follows: 16S rRNA, 5'-AGT-3' and 5'-AGCGTTGCCCCACTTCAAT-3'; fur, 5'-TCCTAGGCTGTG-3' and 5'-TCCACT TTGAAAAATCGTCAA-3'; katA, 5'-T CCTACT TTGAAAAACATCGA-3' and 5'-TACAACTGCCACCTTCCATCA-3'; 33; and 34. As a first step in counterselection mutagenesis, *H. pylori* rpsL mutants were generated by transformation of *H. pylori* with a plasmid containing a cloned *H. pylori* rpsL gene harboring an A-to-G mutation at codon 43 of rpsL (34). This mutation results in a Lys (K)-to-Arg (R) amino acid substitution at position 43, and *H. pylori* strains bearing this mutation are streptomycin resistant. The Sm' rpsL-K43R mutants were then transformed with pfur:cat-rpsL, a nonreplicating plasmid that allows the insertion of a cat-rpsL cassette into the fur open reading frame (ORF). This cassette confers resistance to chloramphenicol mediated by the chloramphenicol acetyltransferase (cat) gene from Campylobacter coli and susceptibility to streptomycin mediated by the intact rpsL gene from *H. pylori* 26695. To generate the pfur:cat-rpsL plasmid, primers 5'-TTACCCGCGA TGTTATTACGGGCTA-3' and 5'-GGCGATAAAACGGCCTTGTT-3' were utilized to PCR amplify a DNA product from *H. pylori* 7.13 genomic DNA extending from approximately 300 bp upstream of the fur translational start site to 900 bp downstream of the fur translational start site. This PCR product was cloned into pGEM-T Easy (Promega), and the resultant fur-containing plasmid was used as a template for inverse PCR with primers 5'-CGACCGCTTCCCGAGGTCTC-3' and 5'-GGCTATGAGAATTTCCGCTTAAGG-3' to facilitate the insertion of the cat-rpsL cassette (33). After ligation with the cat-rpsL cassette, the resultant plasmid (pfur: cat-rpsL), which is unable to replicate in *H. pylori*, was transformed into the rpsL-K43R mutants of *H. pylori* strains 7.13 and RSO251, and single colonies resistant to chloramphenicol (5 μg/ml) but sensitive to streptomycin (25 μg/ml) were selected. Introduction of the cat-rpsL cassette into the fur genes of strains 7.13 and RSO251 was confirmed by PCR amplification and DNA sequencing.

A fur-containing plasmid was used as the template for targeted mutagenesis with the Quik-Change mutagenesis kit (Agilent Technologies). The introduction of mutations within the codon encoding Fur amino acid 88 was confirmed by DNA sequencing. These mutated plasmids were then used to transform *H. pylori* strains harboring fur:cat-rpsL. Transformants were plated onto BB-FBS agar plates containing streptomycin (25 μg/ml) and streptomycin-resistant colonies were isolated. DNA sequencing was used to confirm the loss of the cat-rpsL cassette and the introduction of the desired mutations into the fur ORF.

**Growth of *H. pylori* fur mutants under oxidative-stress or high-salt conditions.** *H. pylori* strains harboring wild-type (WT) fur or fur-fur-R88H were first tagged by the introduction of either a cat or an aph3 cassette into the ureA locus of *H. pylori*. This was carried out with plasmids PAD-C and PAD-K, which contain cat and aph3 cassettes, respectively, flanked by *H. pylori* ureA sequences (35). Proper introduction of the cassettes into the ureA locus was confirmed by PCR analysis and DNA sequencing. We have shown previously that insertion of antibiotic cassettes into this locus does not interfere with *H. pylori* growth (31). To test the susceptibility of *H. pylori* to oxidative-stress conditions, strains (each at 2.5 × 10^7 cells/ml) containing the WT fur or fur-fur-R88H allele and expressing different antibiotic markers were coinoculated into brucella broth supplemented with 5 or 10 μM paraquat (Sigma-Aldrich) as an inducer of oxidative stress (36) or brucella broth without added paraquat. Following overnight growth to an OD600 of ~0.5, serial dilutions of the mixed cultures were plated on BB-FBS plates containing either chloramphenicol or kanamycin. Viable colonies were counted following 5 days of plate growth to determine the proportion of the mixed culture that harbored either the WT fur or the fur-fur-R88H allele. A similar experimental design was used to compare the sensitivities of the WT and fur-fur-R88H mutant strains to high-salt conditions with brucella broth supplemented with 1.2% NaCl.

**Statistical methods.** The statistical significance of differences was analyzed by either Mann-Whitney test (for nonparametric data) or t test with GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). All tests were two tailed, and a P value of <0.05 was considered significant.

**RESULTS**

**Proteomic analysis of *H. pylori* strains.** Previous work demonstrated that *H. pylori*-infected Mongolian gerbils fed a high-salt...
TABLE 1 Comparative proteomic analysis of *H. pylori* strains

<table>
<thead>
<tr>
<th>Protein(s)</th>
<th>Gene no.</th>
<th>No. of assigned spectral counts in strain indicated</th>
<th>Fold difference from input</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expt 1</td>
<td>Expt 2</td>
</tr>
<tr>
<td>Catalase</td>
<td>HPB8_1087/HP0875</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>FrpB1</td>
<td>HPB8_1088/HP0876</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>FecA1</td>
<td>HPB8_887/HP0686</td>
<td>69</td>
<td>45</td>
</tr>
<tr>
<td>PdxA</td>
<td>HPB8_1634/HP1583</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>65,918</td>
<td>82,551</td>
</tr>
</tbody>
</table>

The bacteria were analyzed in a global proteomic profiling experiment (MudPIT) as described in Materials and Methods. In total, 1,175 proteins were identified.

Two independent experiments were performed, with experiment 1 comparing the protein content of the input strain with that of output strains RSO242 and HSO123 and experiment 2 comparing the protein content of the input strain with that of RSO241 and HSO1.

Proteins for which a minimum of 100 spectral counts were identified in both experiments 1 and 2 were analyzed to detect differences in abundance between strains.

HPB8 gene numbers and the corresponding HP gene numbers in the *H. pylori* 26695 genome are listed.

...diet had a higher incidence of gastric cancer than *H. pylori*-infected gerbils fed a regular-salt diet (22). In addition, infected animals fed a high-salt diet had more severe gastric inflammation, a higher gastric pH, and increased parietal cell loss than those on a regular diet (22). We hypothesized that proliferation of *H. pylori* in the intragastric environment associated with a high-salt diet might lead to the selection of strains that are optimally adapted to these conditions. To test this hypothesis, we analyzed *H. pylori* strains cultured from animals fed a regular or high-salt diet, as well as the input strain, by proteomic and whole-genome sequencing methods. A schematic detailing the experimental design and strains used for each analysis is shown in Fig. 1.

We first analyzed the proteomes of output strains cultured from two animals fed a high-salt diet (HSO1, HSO123) and two animals fed a regular-salt diet (RSO241, RSO242) with that of the input strain (see Table S1 in the supplemental material). The proteins exhibiting the greatest differences in abundance are listed in Table 1. Specifically, the two HSO strains (HSO123 and HSO1) produced higher levels of catalase (37), an iron-regulated outer membrane protein reported to bind heme and hemoglobin (FrpB1 [38, 39]), an iron-dicitrate transport protein (FecA1 [40]), and a protein involved in vitamin B<sub>6</sub> biosynthesis (PdxA; 4-hydroxythreonine-4-phosphate dehydrogenase [41]) than the input and RSO strains. Concordant with the results obtained at the protein level, the katA, frpB1, fecA1, and pdxA transcript levels were significantly higher in HSO1 and HSO123 than in RSO241, RSO242, and the input strain (*P* < 0.05) (Fig. 2). Thus, we detected differential abundances of several proteins and corresponding transcriptional alterations when comparing output strains from animals on a high-salt diet with the input strain and with output strains from animals on a regular diet.

Whole-genome sequence analysis. To identify mutations that might account for the differences detected in the proteomic and transcriptional analyses, we next subjected the *H. pylori* strains to whole-genome sequence analysis. We analyzed four single colonies of the input strain, four single colonies of RSO241, and four single colonies of HSO1. To facilitate the identification of differences among these strains, we aligned the sequences with the genome sequence of *H. pylori* strain B8, a closely related strain whose complete genome sequence is available (32). These analyses revealed a large number of substitution mutations, as well as insertions and deletions, many of which were detected in only a small proportion of the sequence reads from an individual single colony isolate. We focused in particular on the single-nucleotide variants (SNVs, different from reference strain B8) that were detected in 100% of the sequence reads from at least 1 of the 12 sequenced single colonies. Table 2 lists the genetic alterations that were identified by comparing RSO241, HSO1, and the input strain with the *H. pylori* B8 reference.

As shown in Table 2, we identified three nucleotide sites (in genes encoding hypothetical protein HPB8_1463 and catalase and...
upstream of *fecA2*) at which multiple colonies of input strain 7.13 used for these experiments differed from the B8 reference sequence. The nucleotide sequences at two of these sites (HPB8_1463 and catalase) are identical in the B8 reference sequence and a recently deposited strain 7.13 draft genome sequence (GenBank accession no. LAQK00000000) (42). Therefore, input strain 7.13 used in the present study exhibited detectable differences from the reference genome at either two or three sites, depending on which reference genome was used.

Three SNVs (in genes encoding hypothetical protein HPB8_593, DNA Pol exonuclease, and Fur) were detected in all four sequenced single colonies of the HSO1 strain but were not found in the input strain (Table 2), the B8 reference genome, or the 7.13 draft genome sequence (GenBank accession no. LAQK00000000). Two of these SNVs were nonsynonymous substitutions, and the third was a synonymous substitution. Specifically, one of the SNVs resulted in an R-to-H change at amino acid 88 of the Fur protein (R88H), another resulted in a Y-to-C change at amino acid 60 of hypothetical protein HpB8_593 (Y60C), and the third was a synonymous substitution in the gene for HpB8_1139 (DNA Pol exonuclease) (Table 2). We identified two SNVs (compared to the reference B8 strain) that were present in RSO241 and the input strain but absent from HSO1. The first of these mutations, located 29 bp upstream of the mapped *fecA2* transcriptional start site (43), was present in all four single colonies of RSO241 and all single colonies of the input strain (Table 2). The other SNV was located three nucleotides into the ORF of a protein predicted to encode a glycine/serine transferase (HpB8_1463) shown to be important for colonization of the mouse stomach (44). This mutation alters the predicted TTG translational start site to TTA (encoding leucine), which probably prevents translational initiation. This SNV was present in three of the four single colonies of RSO241 and three of the four single colonies of the input strain but was not identified in any of the HSO1 single colonies (Table 2).

### TABLE 2 SNVs and insertions of interest

<table>
<thead>
<tr>
<th>Gene no.</th>
<th>Protein</th>
<th>Position</th>
<th>Reference</th>
<th>SNV or insertion</th>
<th>Input*</th>
<th>RSO241*</th>
<th>HSO1*</th>
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<tr>
<td>SNVs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
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<tr>
<td>HPB8_593/HP0953</td>
<td>Hypothetical</td>
<td>563546</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>HPB8_1139/HP1022</td>
<td>DNA Pol Exo</td>
<td>1117619</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
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<td>Fur</td>
<td>1122559</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>HPB8_1463/HP10102</td>
<td>Hypothetical</td>
<td>1434104</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
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<tr>
<td>Upstream of HPB8_1015/HP0807</td>
<td>FecA2</td>
<td>1001780</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

* Four single colonies of the input strain, RSO241, and HSO1 were analyzed by whole-genome sequencing. The results presented correspond to cases where 100% of the sequence reads in at least one of the sequenced samples contained the SNV or insertion indicated, which differed from the reference genome from strain B8. Shading indicates strains containing the mutation indicated.

<table>
<thead>
<tr>
<th>Insertion</th>
<th>Protein</th>
<th>Position</th>
<th>Reference</th>
<th>SNV or insertion</th>
<th>Input*</th>
<th>RSO241*</th>
<th>HSO1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPB8_1087</td>
<td>Catalase</td>
<td>Between 106433 and 106434</td>
<td>CT</td>
<td>CAT</td>
<td>CAT</td>
<td>CAT</td>
<td>CAT</td>
</tr>
</tbody>
</table>

Adaptation

Since two of the differentially abundant proteins identified in the proteomic analysis (catalase and FrpB1) are encoded by genes adjacent to one another on the *H. pylori* chromosome (39), we initially hypothesized that a mutation in the *katA-frpB* intergenic region might account for the alterations in the abundance of these two proteins. However, nucleotide sequence analysis did not reveal any alteration in the *katA-frpB* intergenic region that could account for the alterations in the abundance of catalase and FrpB1. The frameshift mutation in *katA* presumably accounts for the low levels of catalase detected by proteomic analysis, but we did not detect any frameshift or nonsense mutations in *frpB1* that could account for the differences in FrpB1 levels observed in the proteomic analysis. FrpB1 and two other differentially abundant proteins identified in the proteomic analysis (FecA1 and PdxA) are known to be regulated by Fur (39, 43, 45, 46), and therefore, the nonsynonymous mutation detected in *fur* could potentially account for the observed differences in the abundance of these proteins.

### Genetic analysis of multiple output strains

Genome sequence analysis of four colonies of the input strain revealed that two of the polymorphisms described above (in HPB8_1463 and *katA*) were present in the input bacterial population used for gerbil infection (Table 2). In contrast, the mutations in HPB8_593, HPB8_1139, and *fur* were not found in any of the four sequenced colonies of the input strain (Table 2). The latter mutations could have arisen in vivo during *H. pylori* colonization of Mongolian gerbils, or alternatively, heterogeneity at these sites might have been present in the input strain used for infection of the gerbils at a low level, undetectable by sequencing of four single colonies of the input strain. As one approach for differentiating among these possibilities, we examined whether the mutations shown in Table 2 were present in output strains isolated from additional animals fed a high-salt diet or a regular diet. We used...
gene-specific primers to PCR amplify appropriate DNA sequences from additional output strains (eight from animals on a high-salt diet and eight from animals on a low-salt diet) and sequenced the resulting amplicons.

As shown in Table 3, each of the mutations identified by whole-genome sequence analysis (Table 2) was detected in multiple additional output strains, and a second type of fur mutation, encoding Fur-P45T, was identified in one of the output strains (RSO244). Five output strain lineages (designated A to E) were identified on the basis of the distribution of these mutations (Table 3). One output lineage (designated C) was identical to the input strain (variation B), and the mutations defining this lineage were detected in output strains from four (22%) of the animals. The other lineages were all distinct from the input strain. Since specific mutations (including a synonymous mutation) were identified repeatedly in multiple output strains, this suggests that some of the mutations detected in output strains may have been present in the bacterial population used to infect the gerbils. Recombination (either within the initial input strain population or subsequently within animal stomachs) presumably accounts for the emergence of multiple lineages.

We next analyzed the distribution of strain lineages and individual mutations according to the diet that the Mongolian gerbils were fed. Output lineage C (harboring the same markers as the input strain, variation B) was detected exclusively in strains cultured from animals fed a regular diet (Table 3). In contrast, lineage A was detected mainly in output strains from animals fed a high-salt diet (Table 3). The fur-R88H mutation was found significantly more commonly in HSO strains than in RSO strains, with fur-R88H found in all nine HSO strains, compared to four of the nine RSO strains (P = 0.0294, Fisher’s exact test). The HPB8_1463 and katA mutations were found in five and four of the nine RSO

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. (%) of strains/no. of HSO in category</th>
<th>Inflammation score&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SNV&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Insertion&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fur</th>
<th>HPB8_593</th>
<th>HPB8_1139</th>
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<sup>a</sup> Sections of gastric tissue from animals were scored for severity of total gastric inflammation on a 12-point scale that evaluated acute and chronic inflammation in both the corpus and the antrum (22).

<sup>b</sup> PCR was used to amplify target genes from the input 7.13 strain, nine HSO strains, and nine RSO strains with the gene-specific primers listed in Materials and Methods. “Yes” indicates the presence of SNVs or insertions that differed from the reference genome sequence of strain B8 (described in Table 2). —, no difference from reference strain B8.

<sup>c</sup> On the basis of the results in the top portion of the table, H. pylori strains (input and output) were grouped according to the distribution of the mutations identified. The number of strains and the percentage of the total number of strains in each group are presented. In addition to the total number and the percentage of the total number, the number of HSO strains belonging to each category is shown.

<sup>d</sup> Instead of an R88H mutation, Fur in strain RSO244 contained a P45T mutation.
strains, respectively, but in none of the HSO strains \( (P = 0.0294\) and \( P = 0.0824\), respectively). The uneven distribution of mutations between RSO and HSO strains suggests that differential selective pressures led to the emergence of strains adapted to intragastric conditions associated with either the regular-salt or high-salt diet.

**Analyses of catalase nucleotide sequences, enzymatic activity, and transcript levels.** Proteomic analysis of strains HSO1 and HSO123 compared to strains RSO241 and RSO242 and the input strain revealed several differences (Table 1), including a difference in the abundance of catalase. Specifically, levels of catalase were markedly higher in strains HSO1 and HSO123 than in the input, RSO241, or RSO242 strain. Three of the four single colonies of the input strain and three of the four single colonies of RSO241 contained a frameshift mutation in \( \text{katA} \) (Table 2), which presumably accounts for the low levels of catalase detected by proteomic analysis. In comparison to the parental B128 human isolate and several other minimally passaged \( H. \) pylori human isolates, input strain 7.13 demonstrated about 200-fold lower levels of catalase activity (data not shown).

To further investigate heterogeneity in catalase production among output strains, we analyzed the catalase activity in the strains analyzed by proteomic analysis, as well as output strains from additional animals. Overall, the HSO strains demonstrated higher levels of catalase activity than the RSO strains \( (P = 0.044\), Mann-Whitney test) (Fig. 3A). All nine HSO strains had markedly higher catalase activity \((\sim 40\)-fold higher\) than the input strain (Fig. 3A). Four of the nine RSO strains had catalase activity that was similar to that of the input strain, while the remaining strains exhibited catalase activity 20–50-fold higher than that of the input strain. The four RSO strains with low catalase activity (RSO241, RSO242, RSO251, and RSO270) (see Fig. S1A in the supplemental material), similar to that of the WT strain, each harbored a frameshift insertion/mutation in the \( \text{katA} \) gene (Table 2). This frameshift mutation was not detected in the remaining 14 strains (5 RSO and 9 HSO strains) with higher catalase enzymatic activity.

We next examined catalase transcript levels in the 18 output strains (Fig. 3B; see Fig. S1). There was no significant difference in \( \text{katA} \) transcript levels between the HSO and RSO strains (Fig. 3B, \( P = 0.258\), Mann-Whitney test). Among the RSO strains, the four that contained a catalase frameshift mutation \((\text{i.e., RSO241, RSO242, RSO251, and RSO270})\) had low \( \text{katA} \) transcript levels, similar to that of the input strain (see Fig. S1). The other RSO strains with intact \( \text{katA} \) ORFs \((\text{RSO1, RSO243, RSO244, RSO263, and RSO266})\) had transcript levels, on average, about 3-fold higher than that of the input strain and similar to those of HSO strains (see Fig. S1). These results suggest that the \( \text{katA} \) frameshift mutation results in a reduction in \( \text{katA} \) transcription or \( \text{katA} \) transcript stability. In addition, these results suggest that there was selection for strains with a high level of catalase activity in the gastric environment associated with a high-salt diet.

**Analysis of \( \text{frpB1}, \text{fecA1}, \) and \( \text{pdxA} \) transcript levels.** The proteomic analysis revealed marked differences in the abundance of FrpB1, FecA1, and PdxA among the strains analyzed (Table 1). To further investigate this heterogeneity, we analyzed the transcription of \( \text{frpB1}, \text{fecA1}, \) and \( \text{pdxA} \) in the strains examined by proteomic analysis, as well as additional output strains (Fig. 3C to E). All of the HSO strains had \( \text{frpB1}, \text{fecA1}, \) and \( \text{pdxA} \) transcript levels that were higher than those of the input strain. In contrast, only five of the nine RSO strains \((\text{RSO1, RSO243, RSO244, RSO263, and RSO266})\) (see Fig. S1) had \( \text{frpB1}, \text{fecA1}, \) and \( \text{pdxA} \) transcript levels higher than those of the input strain; the remaining four RSO strains \((\text{RSO241, RSO242, RSO251, and RSO270})\) had transcript levels similar to those of the input strain. Compared to the RSO strains as a group, the HSO strains had higher \( \text{frpB1} \) \((P = 0.032), \text{pdxA} \) \((P = 0.041), \) and \( \text{fecA1} \) \((P = 0.136)\) transcript levels (Fig. 3C to E). Thus, the transcriptional profiles of output strains from animals on a high-salt (HSO) diet compared to those of input strain 7.13. Each data point represents the mean catalase activity or mean transcript level of each of the 18 output strains. The mean catalase activity and mean relative transcript level of each strain were calculated on the basis of at least three independent experiments. \( P \) values were calculated with the Mann-Whitney test.

**Correlation between \( \text{fur} \) mutations and \( \text{katA} , \text{frpB1}, \text{fecA1}, \) and \( \text{pdxA} \) transcript levels.** Nucleotide sequence analyses (Tables 2 and 3) revealed the presence of a nonsynonymous \( \text{fur} \) mutation in all of the HSO strains, and previous studies have shown that Fur regulates the expression of \( \text{frpB1}, \text{fecA1}, \) and \( \text{pdxA} \) \((39, 43, 45–48)\). Therefore, we hypothesized that the variations in Fur observed among the output strains \((\text{i.e., the WT [Fur-R88], Fur-R88H, or Fur-P45T})\) might account for the differences in \( \text{frpB1}, \text{fecA1}, \) and
Materials and Methods) to introduce a fur-R88H mutation into output strain RSO251 (which contains a WT fur gene encoding an R88 form of Fur) (Fig. 5A). In parallel, as a control, we used the same protocol to manipulate strain RSO251 and restore the original WT R88 form of Fur (Fig. 5A). Higher expression of frpB1, fecA1, and pdxA was detected in the R88H mutant (shown in Fig. 5 as fur-R88H-1) than in the original RSO251 strain containing WT fur (Fig. 5B to D). As expected, the manipulated RSO251 strain in which the WT fur allele was restored (shown as WT fur-1) showed little or no increase in frpB1, fecA1, and pdxA transcript levels compared to the original WT RSO251 strain. The frpB1 and pdxA transcript levels of the fur-R88H-1 strain were significantly higher than those of the strain with restored WT Fur (P = 0.027) (Fig. 5B and C) but were lower than the corresponding levels detected in a Δfur mutant. No difference in the expression of katA (Fig. 5E) or the housekeeping gene (gyrB for DNA gyrase) (Fig. 5F) was detected when the fur mutant strains were compared with strains containing WT fur, indicating that Fur does not regulate the expression of either katA or gyrB. A similar mutagenesis approach was carried out with input strain 7.13 (which encodes WT Fur-R88), and similar results were obtained (Fig. 5G to I). These data indicate that the association between the fur-R88H mutation and katA transcript levels shown in Fig. 4 is not directly attributable to an alteration in fur but instead is attributed to the frameshift mutation in katA that was present in the four RSO strains containing WT fur.

As another approach to assess the effect of the R88H mutation on frpB1 expression, we reintroduced the WT fur allele into the RSO251 strain encoding Fur-R88H (i.e., fur-R88H-1, Fig. 6) as described in Materials and Methods and the legend to Fig. 6. The frpB1 transcript levels in strains designated WT fur-1, fur-R88H-1, WT fur-2, fur-R88H-2, and Δfur1 (Fig. 6A) were compared to that in the original RSO251 output strain. Consistent with the results shown in Fig. 5, the strain harboring the mutated fur-R88H allele (strain fur-R88H-1) expressed higher levels of frpB1 than the WT fur-1 strain (P < 0.05), but the levels of frpB1 expression were lower than those detected in Δfur1 (the fur null mutant) (Fig. 6B). When WT fur was introduced into the Δfur1 null mutant strain (resulting in WT fur-2), frpB1 transcript levels were reduced to levels similar to those found in the WT fur-1 strain. In contrast, when fur-R88H was introduced into the Δfur1 mutant (resulting in fur-R88H-2), frpB1 transcript levels were similar to those found in strain fur-R88H-1. These results provided further evidence that the Fur-R88H mutation influences the levels of frpB1 transcription.

Strains producing Fur-R88H are more resistant to salt and oxidative stress. Previous studies have shown that H. pylori fur mutant strains demonstrate a growth defect at elevated salt concentrations compared to the WT strain (49), and several Fur-regulated genes (e.g., napA and sodB) are involved in oxidative-stress resistance in H. pylori (50–54). Therefore, we hypothesized that the fur-R88H mutation might affect the ability of H. pylori to grow in either an oxidative-stress environment induced by paraquat (36) or medium containing elevated salt concentrations. In initial experiments (Fig. 7A), we introduced a kanamycin resistance marker (aph3) into strain RSO251-WT fur-1 (producing WT Fur) and a chloramphenicol resistance marker (cat) into strain RSO251-fur-R88H-1 (Fig. 6A). To allow simultaneous and direct comparison of the effects of either NaCl or paraquat on WT and fur-R88H bacteria, a condition reflective of the competition.
that occurs within the stomach, we performed competition assays. The marked strains were coinoculated (at equivalent optical densities) into three liquid media: brucella broth without additives, brucella broth with an elevated NaCl concentration (1.2% NaCl), and brucella broth containing paraquat. After growth overnight, the cultures were plated on medium containing kanamycin or chloramphenicol to select for bacteria containing WT fur or fur-R88H, respectively, and the numbers of CFU per milliliter of culture were determined. As shown in Fig. 7A, the composition of the bacterial population from overnight cultures in brucella broth without additives was about 55% fur-R88H (2.8 × 10⁷ CFU/ml) and 45% WT fur (2.1 × 10⁷ CFU/ml). When the bacteria were cultured in brucella broth containing paraquat, fur-R88H bacteria constituted about 90% of the population (P < 0.05) (Fig. 7A). Bacteria harboring WT fur and fur-R88H were each susceptible to the effects of paraquat, leading to a reduction in the numbers of viable bacteria (3.5 × 10⁶ CFU/ml fur-R88H and 2.7 × 10⁵ CFU/ml WT fur cultured in paraquat-containing medium). When the coculture experiments were performed with high-salt medium, the fur-R88H containing bacteria again made up about 90% of the population (P < 0.05) (Fig. 7A). Bacteria harboring WT fur and fur-R88H were each susceptible to the effects of paraquat, leading to a reduction in the numbers of viable bacteria (3.5 × 10⁶ CFU/ml fur-R88H and 2.7 × 10⁵ CFU/ml WT fur cultured in paraquat-containing medium). When the coculture experiments were performed with high-salt medium, the fur-R88H containing bacteria again made up about 90% of the population (P < 0.05) (Fig. 7A).
In this study, we tested the hypothesis that the evolution of *H. pylori* within a host can be influenced by the composition of the diet. Specifically, we hypothesized that the altered gastric environment generated by a high-salt diet might promote the selection of *H. pylori* strains adapted to these gastric conditions. To test this hypothesis, we analyzed *H. pylori* strains isolated from experimentally infected Mongolian gerbils that were fed either a high-salt diet or a regular-salt diet over a 4-month time period. Proteomic, transcriptional, and nucleotide sequence analyses revealed multiple differences between output strains from animals fed a high-salt diet and output strains from animals on a regular-salt diet.

The mutations detected in the output strains (Table 2) could potentially have arisen *in vivo*. Alternatively, since we detected evidence of genetic heterogeneity in the *H. pylori* population that was administered to gerbils, some of the mutations were probably already present in the *H. pylori* input population prior to the start of the animal experiments. Regardless of the time point when these mutations first arose, the results of this study provide evidence that there was positive selection of specific *H. pylori* variants during the course of infection. We propose that certain variants have increased fitness for proliferation in the gastric environment associated with a high-salt diet than in the gastric environment associated with a regular-salt diet. As described previously, these two types of gastric environments differ in multiple features (22).

In addition to the differences in gastric luminal salt concentrations, the *H. pylori*-infected animals fed a high-salt diet had lower levels of gastric acidity and higher levels of gastric inflammation than infected animals fed a regular-salt diet (22). At present, it is not possible to determine whether the high-salt diet was directly responsible for the observed selection of *H. pylori* variants or whether the effect of a high-salt diet was indirect (for example, due to different levels of gastric inflammation or differences in pH). Similarly, at present, it is not possible to determine if there was a single dominant selective pressure or if the selection resulted from a combination of multiple environmental pressures. Human diets vary considerably in salt content (as well as in many other ways). Therefore, we presume that similar selection of *H. pylori* variants occurs continually in humans in response to the composition of their diet. The present results suggest that variation in the composition of the human diet may be an important factor that influences the evolution of *H. pylori* in individual human stomachs.

Proteomic analysis of representative strains revealed higher levels of KatA, FrpB1, FecA1, and PdxA in the HSO strains than in the RSO strains and the input strain. The differences in the abundance of FrpB1, FecA1, and PdxA were attributable to transcriptional differences, and the difference in the abundance of KatA was attributable primarily to a frameshift mutation in the catalase gene. Genome sequence analysis of representative strains revealed the presence of multiple polymorphisms in an HSO strain compared to RSO and input strains, including a *fur-R88H* mutation, an alteration in the catalase gene resulting in the correction of a frameshift mutation, a mutation encoding a Y60C substitution in the cosyltransferase, HPB8_1463. These mutations were distributed nonrandomly in HSO strains compared to RSO strains (Table 2). For example, the *fur-R88H* mutation was identified in HSO strains significantly more commonly than in RSO strains.

**FIG 7** Effects of paraquat (PQ) and an increased sodium chloride concentration on the growth of *H. pylori* strains harboring WT fur or the *fur-R88H* allele. RSO251 strains harboring either WT fur (*fur-1*) or the *fur-R88H*-1 allele were generated as shown in Fig. 5A. The bacteria were then tagged by the introduction of antibiotic markers (*cat* [chloramphenicol acetyltransferase] or *aph3* cassette) into the *ureI* locus. Competition assays were performed by inoculating equal numbers of the two bacterial strains into medium containing paraquat (5 or 10 μM) or 1.2% NaCl and culturing them for 15 h. The cultures were then plated on BB-FBS plates containing either kanamycin or chloramphenicol. Panel A illustrates results of competition experiments in which a strain harboring the WT fur allele was tagged with an *aph3* cassette and a strain harboring the *fur-R88H* allele was tagged with a *cat* cassette. Panel B illustrates results of experiments in which a strain harboring the WT fur allele was tagged with a *cat* cassette and a strain harboring the *fur-R88H* allele was tagged with an *aph3* cassette. Results represent the mean ± the standard error of the mean based on at least three independent experiments. Percentages of colonies producing either WT Fur or Fur-R88H after overnight selection are shown. *P < 0.05 (Student’s t test). NT, no treatment.
Experimental studies provided evidence that the Fur-R88H mutation accounts for the altered expression of frpB1, fecA1, and pdxA. Like Fur in other bacterial species, H. pylori Fur can, in the presence of ferrous iron, repress transcription by binding fur box sequences upstream of target genes. frpB1, fecA1, and pdxA are examples of H. pylori genes repressed by iron-bound Fur (39, 43, 45–48). The Fur R88H and P45T mutations are each located close to Fur amino acid residues important for iron coordination of Fur (E90 and H42, respectively) (55), and therefore, we speculate that these mutations might affect the ability of Fur to regulate its target genes. Consistent with this hypothesis, several mutations close to Fur R88 (e.g., R87Q, Y89C, and E90Q) are known to impair Fur regulatory activity (56). Although substitution mutations at the Fur R88 position are not found commonly in H. pylori isolates from humans, an R88H mutation identical to the mutation observed in the present study has been detected in at least three H. pylori isolates from humans (57).

Previous studies have shown that H. pylori Fur has a role in resistance to high-salt conditions (49) and resistance to oxidative stress (50–54) and contributes to H. pylori colonization of the stomach (58). To further investigate the functional consequences of the Fur-R88H mutation, we compared the abilities of H. pylori strains producing either WT Fur or Fur-R88H to grow in medium containing elevated salt concentrations or in oxidative-stress environments. Under both conditions, H. pylori strains expressing Fur-R88H exhibited a competitive advantage over strains expressing WT Fur. The exact mechanisms by which the R88H mutation influences these two phenotypes is not yet known, but there are several likely possibilities. For example, since Fur-R88H-producing strains express higher levels of iron transport proteins FrpB1 and FecA1, we speculate that these strains might acquire iron more efficiently than strains producing WT Fur. Since iron functions as a cofactor for oxidative-stress enzymes such as catalase and SodB, increased iron uptake in Fur-R88H-producing strains might lead to increased survival of H. pylori under oxidative-stress conditions. The mechanisms by which the R88H mutation contributes to increased salt resistance are less clear. Previous studies of Bacillus subtilis and B. cereus suggested a link between bacterial stress responses to high-salt conditions and oxidative stress (59, 60). For instance, salt stress has been reported to induce the production of proteins that allow cross-protection against both oxidative stress and salt stress (59, 60). Thus, it is possible that H. pylori utilizes the same stress response when it encounters either elevated salt concentrations or oxidative stress. This idea is supported by the fact that Fur has been proposed to be a global regulator in H. pylori, responding to diverse stimuli such as iron limitation, low pH, oxidative stress, and salt stress (46, 49, 51). Alternatively, exposure of the bacteria to high salt concentrations could result in oxidative stress (59) or could affect the bioavailability of iron to the bacteria. For example, studies with Bacillus have shown that high-salt conditions reduced iron availability, resulting in the upregulated expression of genes required for siderophore synthesis and iron uptake systems, through a Fur-dependent process (61). Moreover, high-salt conditions retarded B. subtilis growth, an effect that was partially reversed by the addition of excess iron to the growth medium (61). An enhanced ability of the strain containing Fur-R88H to scavenge iron through increased expression of iron uptake enzymes might confer a survival advantage under high-salt conditions. The consumption of high levels of dietary salt has been associated with an increased risk of gastric cancer in human populations (14), and we demonstrated previously that H. pylori-infected Mongolian gerbils fed a high-salt diet were more likely to develop gastric cancer than were infected animals fed a regular-salt diet (22). The present results provide evidence that the evolution of H. pylori is influenced by the composition of the diet and that a high-salt diet leads to the selection of strains that differ from strains present in animals on a regular-salt diet. Only a small number of mutations were identified in the present study because of the relatively short time period in which animals were fed the different diets and because output strains from only two animals were subjected to whole-genome sequence analysis. Presumably, the composition of the diet would have an even greater detectable impact on the evolution of H. pylori if longer periods of time and strains from larger numbers of animals were studied. The most striking genetic change detected in the present study resulted in altered levels of proteins involved in iron acquisition and oxidative-stress resistance, but the composition of the diet could potentially have a much broader impact on the evolution of H. pylori. We speculate that the adaptation of H. pylori strains to the gastric environment associated with a high-salt diet may concomitantly result in the emergence of strains that have an increased carcinogenic potential.

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