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Point Mutations in *Helicobacter pylori*’s *fur* Regulatory Gene that Alter Resistance to Metronidazole, a Prodrug Activated by Chemical Reduction

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**Abstract**

*Background:* *Helicobacter pylori*’s Fur regulatory protein controls transcription of dozens of genes in response to iron availability, acidity and oxidative stress, and affects the vigor of infection and severity of disease. It is unusual among Fur family proteins in being active both when iron-loaded and iron-free.

**Methodology/Principal Findings:** We tested if *H. pylori* fur mutations could affect resistance to metronidazole (Mtz), an anti-*H. pylori* prodrug rendered bactericidal by chemical reduction. Point mutations were made by PCR in DNA containing *fur* and a downstream chloramphenicol resistance gene, and were placed in the *H. pylori* chromosome by transformation of a *fur-deletion* (Δ*fur*) strain. Several substitutions affecting *H. pylori* Fur’s ~10 residue N terminal arm, which has no counterpart in prototype (*E. coli*-type) Fur proteins, increased Mtz resistance, as did mutations affecting the region between DNA binding and dimerization domains. Three types of mutations decreased resistance more than did Δ*fur:* substitutions affecting the N-terminal arm; substitutions affecting the metal binding pocket; and nonsense mutations that resulted in a truncated Fur protein with no C-terminal dimerization domain. Most metal binding pocket mutations were obtained only in *fur* genes with additional inactivating mutations, and thus seemed deleterious or lethal because they.

**Conclusions/Significance:** These results establish that *H. pylori* Fur’s distinctive N terminal arm is functional, and more generally illustrate that point mutations can confer informative phenotypes, distinct from those conferred by null mutations. We propose that *fur* mutations can affect Mtz susceptibility by altering the balance among Fur’s several competing activities, and thereby the expression of genes that control cellular redox potential or elimination of bactericidal Mtz activation products. Further analyses of selected mutants should provide insights into Fur interactions with other cellular components, metabolic circuitry, and how *H. pylori* thrives in its special gastric niche.

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**Introduction**

The gastric pathogen *Helicobacter pylori* chronically infects the stomachs of billions of people worldwide. It is highly specific for gastric epithelial cell surfaces and a narrow band of overlying mucous, an inherently unstable niche that is hostile to other microbial species [1–3]. *H. pylori* infections typically start in early childhood, can last for life if not treated, and constitute a major cause of gastric and duodenal ulcer disease and gastric cancer. Persistent infection is thought to depend on a constellation of quantitative factors – prominent among them, *H. pylori*’s abilities:

- (i) to provoke low level tissue damage and inflammation and the release of nourishing metabolites, without destroying the gastric epithelium on which *H. pylori* depends;
- (ii) to cope with inflammation-associated oxidative stresses and transient exposure to stomach acid;
- (iii) to acquire iron and other micronutrients needed as metalloprotein cofactors and protein structural components, while avoiding the toxicity of these metals when they are in excess or not properly sequestered; and
- (iv) to swim away from acidic environments and toward the near-neutral, nourishing epithelial surface [1–7].

Each of these activities is affected or controlled in part by the *H. pylori* Fur protein, which belongs to a widespread family of transcription regulators whose members have been most studied in terms of controlling iron uptake and utilization [4–6,8]. Prototype Fur proteins, such as that of *Escherichia coli*, act as simple repressors when complexed with ferrous iron and are inactive when iron-free. Their iron-bound forms (Fe-Fur) directly block transcription of some target genes (e.g., for iron uptake) and increase transcription of other genes (e.g., for iron storage), whereas their iron-free forms (Apo-Fur) are inactive. In these cases, positive regulation by Fe-Fur is indirect – via repression of transcription of a gene whose product, in turn, represses target genes.
The Fur protein of *H. pylori* seems more complex functionally than the prototypes: Apo-Fur and Fe-Fur each bind cognate regulatory site DNAs; and each represses transcription of certain genes and apparently stimulates transcription of others. Furthermore, *H. pylori* Fur autoregulates its own synthesis – inducing synthesis when iron is limiting by binding to one regulatory DNA sequence, and repressing synthesis when iron is abundant by binding to a nearby sequence (Fig. 1) [4–6]. We hypothesize that *H. pylori* Fur has two distinct active conformations: Fe-Fur (Fig. 2A), which binds to one set of DNA sequences, and a distinct Apo-Fur form, which binds to other operator sequences (see for example ref 11). Positive regulation by *H. pylori* Fur is thought to be direct [4–6] – formally equivalent to that by λ CI repressor [12], although the possibility of indirect control is raised again by the finding of many small non-coding regulatory RNAs in *H. pylori* [13]. X-ray crystal structures of several Fur and Fur-type regulatory proteins complexed with zinc or manganese have been determined [9,11,14–17].

Several mutational changes in *H. pylori* Fur proteins that affect parameters such as repression mediated by Fe-Fur or Apo-Fur, iron loading, sodB (superoxide dismutase) expression and metronidazole (Mtz) resistance and/or protein dimerization were described recently [18,19]. Although the structure of a Zn-bound form of a mutated but still active form of *H. pylori* Fur has been determined recently [16], much still remains to be learned about this protein’s functionally important residues and conformations, especially those of Apo-Fur and the forms that mediate responses to acid or oxidative stress.

The present experiments were begun after finding a mutation in *fur* gene codon 3 (change of Arg to Ile; “*furR3I*”) in an *H. pylori* strain that had undergone multiple steps of mutation and selection for increased resistance to metronidazole (Mtz) [20]. This mutation affected *H. pylori* Fur’s N-terminal arm, a ~10 amino acid long segment with no counterpart in Fur proteins that have been structurally characterized previously, and whose role, if any, had not been assessed previously. The potential importance of N-terminal arms, however, had been well documented in early studies of phage λCI and *E. coli* AraC repressor proteins [21–23]; and N-terminal arms similar in length to *H. pylori* Fur’s, but divergent in sequence, are found in Fur proteins of *H. pylori*-related species (Fig. 2C). An analogous N-terminal arm is also found in *H. pylori’s* nickel-responsive NikR regulatory protein, a feature whose presence and composition also varies widely among related proteins [24], and that affects DNA binding specificity [25]. Accordingly, we hypothesized that *H. pylori* Fur’s N-terminal arm is functional, and that the appearance of the *furR3I* mutation stemmed from its enhancement of Mtz resistance. Mtz exposure is mutagenic [26] however, and a *fur* mutation had appeared in only one of the two highly resistant lineages that were analyzed [20]. Hence, an alternative interpretation was that *furR3I* might be a bystander, and that the observed increase in Mtz resistance had been caused by mutation at another unknown locus.

It is with this background, interest in novel features of multifunctional regulatory proteins such as Fur and survival strategies of niche-specialists such as *H. pylori*, and with the long-range goal of better understanding *H. pylori* Fur’s structure, function and regulatory circuitry, that we carried out the mutational analysis of *H. pylori* fur described below.

**Results**

The *furR3I* point mutation and a *fur* deletion each affect Mtz resistance, but differently

To critically test if the *furR3I* allele does increase Mtz resistance, a chloramphenicol resistant gene (*cat*) was inserted downstream of a wild-type (*wt*) *fur* gene by a direct PCR method [27,28]. The resultant PCR product was used to transform *H. pylori* strain M1.5 (Fig. 3A), which contains *furR3I* and also four other mutations that had appeared earlier in our multistep selection for high level Mtz resistance (in genes *rdxA, frxA, ribF* and *mldA*) [20]. Dilution and plating tests showed that single cells of strain M1.5 reproducibly formed colonies with 100% efficiency on agar medium containing 230 μg of Mtz/ml, but were killed (~1% survival) on agar containing 250 μg of Mtz/ml (phenotype designated 230R,250S) [20,29]. Chloramphenicol resistant (Cam’) transformants of strain M1.5 were selected, and *fur* genes of several of them were PCR amplified and sequenced. Those transformants that had received the *fur*-set allele had a 190R,220S phenotype, and were less Mtz resistant than isogenic Cam’ siblings that had retained *furR3I* (230R,250S) [20]. These quantitative differences in Mtz resistance phenotypes were seen reproducibly in side-by-side comparisons on the same petri plates, and in repeated efficiency of plating tests. This outcome established that *furR3I* does indeed contribute to strain M1.5’s high level Mtz resistance.

In parallel, PCR products were generated in which the *fur* gene was replaced by a kanamycin resistance determinant (*aphA*) (Δ*fur-aphA* allele), or simply deleted in PCR products containing a downstream *cat* gene (Δ*fur-cat* allele). Kan’ or Cam’ transformants of strain M1.5 made with these constructs were less Mtz resistant (phenotype 160R,190S) than were those containing the *fur*-set allele (190R,220S) (Fig. 4A). The finding that *A* fur decreases resistance whereas *furR3I* increases resistance, relative to *fur*-set, further emphasizes that *H. pylori* Fur protein’s N-terminal arm is functional.

![Figure 1](https://example.com/figure1.png)

**Figure 1. Positive and negative transcriptional regulation by *H. pylori* Fur protein when complexed with iron (Fe-Fur) and when free of iron (Apo-Fur).** Based on refs 4–6.

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Mutations in N-terminal arm can variously decrease or increase resistance

We had found that reference strain 26695, the wild type ancestor of strain M1.5, is exquisitely sensitive to low levels of Mtz (1R,3S in phenotype) [29,30], and that strain M1.5's high level Mtz resistance could be increased further by additional cycles of selection on agar with higher levels of Mtz (D Dailidiene and DE Berg, unpublished). This outcome and the different resistance levels conferred by Fur-wt, furR3I alleles in strain M1.5 indicated that this strain would be well suited for scoring

![Diagram of H. pylori Fur dimer with mutations highlighted.](image)

**Figure 2. Mutational changes in H. pylori Fur that affect Mtz resistance in distinct regions of the protein.**

A) Structure of the Zn-loaded *H. pylori* Fur dimer [9] (PDBID 2XIG). This shows mutated residues and those of the Fe-binding pocket (H42, E90, H97, H99, E110), secondary (2u; H96, D98, E117, H134) and structural Zn-sites (C102, C105, C142, C145) [9]. This image was generated using Chimera [10]. B) A rotated view (-90°) of the Fe-binding site of a Fur monomer subunit. Protein side chains are colored by atom (red, oxygen; blue, nitrogen; yellow, sulfur; and gray, carbon). Metal ions where visible are colored purple. The positions of three classes of mutations identified in this study are indicated: DNA-binding domain – E8, Y65, K57; DNA binding domain-iron binding pocket/dimerization domain interface – R3, G40, T41, E95; and Fe-binding pocket (see numbering above). C) Sequence alignment of *H. pylori* strain 26695 Fur protein with Fur sequences from other selected bacterial species. Residues that were mutated in this study are indicated with bold numbers and letters. Conserved metal- or DNA-binding ligands are boxed and their functional roles are indicated above the alignment. The primary Fe-site residues (bold outline) are based on the structure of Zn-loaded *HpFur*, but have not been demonstrated crystallographically for any Fur protein. The structural Zn-site is not conserved amongst Fur family members.

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Mutations in N-terminal arm can variously decrease or increase resistance

We had found that reference strain 26695, the wild type ancestor of strain M1.5, is exquisitely sensitive to low levels of Mtz (1R,3S in phenotype) [29,30], and that strain M1.5's high level
phenotypic effects of other fur alleles over a large dynamic range. We therefore used the Δfur-aphA derivative of M1.5 as a recipient strain for fur DNA transformation and analyses described below.

Additional mutations at codon 3 were made using the two-step PCR protocol diagrammed in Fig. 3B to learn if changes at this position that affected Mtz resistance would increase it. A first PCR was carried out with primers designed to generate a near-random set of codon 3 mutations (primers #2 and #3) in combination with upstream and downstream flanking primers (#1 and #4, respectively). This generated two sets of PCR products that overlapped by 44 bp, and that each contained many codon 3 changes. A second PCR using these two products and only the flanking primers (#1 and #4) generated a population of full length fur-cat DNA products that was then used to transform strain M1.5 Δfur-aphA (Fig. 3C). All Cam’ transformants were Kan’, as expected of replacement of the Δfur-aphA allele. Eight transformants carrying new codon 3 alleles but no other mutations elsewhere in fur were identified by DNA sequencing, and were tested quantitatively for levels of Mtz resistance. Two mutations, furR3S and furR3N, decreased Mtz resistance more than did the Δfur alleles – at the limit, conferring a 64R,90S phenotype, in contrast to the 160R,190S phenotype conferred by Δfur; one
mutation, furR3K, increased resistance (220R, 230S) almost as much as did the original furR3I mutation (230S, 250R), and five others conferred levels of resistance similar to those of the Fur or fur-wt alleles (furR3G, R3D, R3T and R3P, R3L, respectively) (Fig. 4A). A mutation of fur codon 8 (furE8K) had been found in a separate direct selection for increased Fur resistance (D Dailidiene and DE Berg, unpublished). DNA containing the furE8K allele was tagged with the downstream cat gene (Fig. 3A) and used to transform strain M1.5Dfur-aphA. Like furR3I, this furE8K allele conferred a 220R, 230S phenotype in the strain M1.5 background, indicating that Fur N-terminal arm position 8 is also important functionally. A deletion of fur codons 2 through 7 (D2–7 in Fig. 4A) was made similarly. It conferred a Mtz resistance phenotype equivalent to that of the total fur gene deletion (Dfur) (Fig. 4A).

Collectively, these results show further that H. pylori Fur’s distinctive N-terminal arm is functional. Because some position 3 changes increased resistance, whereas others decreased resistance more than did simple null mutations, we propose that the Fur’s N-terminal arm position 8 is also important functionally.
terminal arm can adopt two different structures and actions, only one of which is indicated in the recently determined structure of zinc-loaded Fur protein [9] presented in Fig. 2.

Induction and direct selection of fur mutations that affect Mtz resistance

Mutations anywhere in fur that increased Mtz resistance were also sought. A DNA fragment containing fur-wt, a downstream cat gene and flanking sequences (Fig. 3A) was PCR amplified in MnCl₂-containing buffer to stimulate DNA synthesis errors, and the product was used to transform strain M1.5 A fur-aphA, as described above. Four transformants selected only for chloramphenicol resistance marker. Tests of transformants verified that each mutation had indeed increased Mtz resistance, relative to the product was used to transform strain M1.5 A fur-aphA, as described above. Four transformants selected only for chloramphenicol resistance marker. Tests of transformants verified that each mutation had indeed increased Mtz resistance, relative to the recipient a fur-wt allele of genes likely to affect cellular reductive potential.

The strain M1.5 derivatives carrying the furH99R,Y65N double mutant allele exhibited a 90R,130S (highly Mtz-sensitive) phenotype. Paradoxically those that also carried the furT41A mutation, which enhanced resistance in other contexts, were even more Mtz-sensitive (50R,64S).

A more general search for non-lethal iron binding pocket mutations caused severe Mtz sensitization (ranging from 3R,8S to 64R,90S, depending on allele) (Fig. 4C). The two most extreme alleles, furH42A and furH42L, also caused slower growth on Mtz-free agar (colony formation from single cells in three rather than two days), whereas the other seven furE90A or H, furE97G, furH99P, or furE110H, P or N) had no obvious effects on colony size or growth rate. Further experiments will be needed to test if the extreme Mtz susceptibility and the poor growth conferred by the extreme Mtz susceptibility and the poor growth conferred by furH42 mutations stem from loss of Fur’s iron-dependent activities, perhaps without much impairment of Apo-Fur activities.

Although mutations furE90A, E110N and Y65N each caused marked Mtz sensitization (16R-50R range), double mutants containing furH99P or E110N, R or P each conferred a Afur-like 160R,190S phenotype (data not shown). Possible explanations include degradation of the double mutant Fur proteins or altered balance of Apo-Fur and Fe-Fur activities.

Mtz sensitization caused by H. pylori Fur truncation

A nonsense mutation in fur codon H99 (designated furH99*), obtained by PCR mutagenesis (Fig. 3), caused severe Mtz sensitization (50R,64S, in contrast to 160R,190S conferred by Afur) (Fig. 4D). In addition, three alleles obtained by mutating fur codon E110 each contained nearby frameshift mutations that resulted in Fur protein truncation at protein position 115 and caused Mtz sensitization (Fig. 4). In contrast, nonsense mutations
at codons 42, 57, 78 and 86 each conferred a 160R,190S phenotype, equivalent to that of A fur alleles. A codon 93 nonsense mutation conferred an intermediate 150R,160S phenotype (Fig. 4D). The similar phenotypes conferred by a A fur allele and by nonsense mutations that caused protein truncation at or before position 93 suggest that Fur remnants of ≤66 amino acids lack all regulatory activity or are degraded. The greater sensitization caused by truncation at codons 93, 99 and 115 would then be explained if the longer Fur remnants retain some Apo-Fur DNA binding activity (despite an inability to dimerize or bind iron effectively) and an ensuing imbalance in Fur-regulon gene expression.

The double mutant alleles furR3S,H99* and furR3D,H99* each conferred a 160R,328S phenotype, which is lower than the resistance phenotypes conferred by any of these three fur mutations alone (Fig. 4C,D). This outcome indicates that the H. pylori Fur N terminal arm’s critical role does not require interaction with the C terminal dimerization domain, and suggests that the arm and dimerization domains have separate (additive) effects on H. pylori’s regulatory circuitry. In addition, the alleles furR3L,H99* and furR3L,H99* conferred a A fur-like (160R,190S) phenotype. Perhaps these latter two changes of residue 3 cause Fur protein inactivation or degradation when in a Fur remnant context; this would be distinct from the changes in gene regulation that they cause when full length Fur proteins.

Epistasis and background genotype impact on Fur-determined phenotypes

Loss of function mutations in the related rdxA and fxa4 nitroreductase genes are responsible for the first two steps in development of Mtz resistance in most wild type Mtx+ H. pylori strains [29,30], and restoration of a functional rdxA gene in strain M1.4 (fur+ parent of M1.5 used here) restored the original Mtx sensitive phenotype nearly completely, despite its mutations in ribF, mdaB and fxa4 (D Dailidiene and DE Berg, unpublished data). This result indicates that the rdxA-att (functional) allele is “epistatic” to (masks effect of) several other mutations that contribute to Mtx resistance. To learn if the changes in resistance phenotype conferred by fur mutations depended on M1.5’s other resistance-associated mutations, recipient strains were prepared by moving the A fur-aphtA allele into the ancestral wild type strain 26695 and into its derivative mutant only in rdxA and fxa4 (“strain M2.2”); contains functional alleles of genes ribF and mdaB. These A fur derivative strains were then transformed with DNA containing informative cat-linked fur alleles.

Neither the A fur nor furR3I alleles markedly affected the very low level of intrinsic Mtx resistance of 26695 wild type (phenotype 1R,3S), in accord with only rdxA-att being epistatic on other resistance gene mutations. In strain M2.2 (null alleles in rdxA and fxa4), however, furR3I caused a mild but reproducible increase in resistance (from 32R,50S to 50R,64S). Resistance was also enhanced by a furT41I mutation (to 64R,90S), and was sharply diminished by the furH99* nonsense mutation (to 8R,12S). The furR3S mutation also decreased resistance in the M2.2 background, albeit rather subtly: like fur-att, furR3S allowed 100% cell survival of M2.2 on agar with 32 μg Mtx/ml, most significant, however, on agar with 50 μg Mtx/ml, the fur-att and furR3S alleles allowed survival of 10⁻⁷ and only 10⁻³, respectively. These differences in survival were seen in repeated tests in which these two isogenic strains, grown in parallel on Mtx-free agar, diluted and aliquots of diluted cultures were spotted on complementary halves of the same plates with 50 μg Mtx/ml in the agar.

The generality of effects seen in these 26695 lineage strains was evaluated further using derivatives of reference strains SS1, X47 and G27, each with null alleles of rdxA and fxa4 and functional wild type alleles of ribF and mdaB (corresponding to the 26695-derived strain M2.2 used above). Resistance was also diminished by the A fur, furR3S and furH99 alleles and enhanced by the furR3I and furT41I alleles in these strain backgrounds (data not shown). Collectively, these results indicate that wild type alleles of ribF and mdaB are not epistatic on fur mutant alleles, and that the regulatory imbalances caused by changing H. pylori Fur’s unique N terminal arm or body are general, not likely to depend on any unique strain genetic background.

Discussion

H. pylori’s Fur protein is a multifunctional regulator that controls transcription of dozens of genes, some negatively and some positively, and variously in response to iron availability, pH and oxidative stress [Fig. 1]. The present mutational analysis was begun after finding a mutation affecting H. pylori Fur’s distinctive N terminal arm after multiple steps of selection for ever higher levels of Mtx resistance, and was motivated by interest in multifunctional regulatory proteins such as Fur and the medical significance of H. pylori and its resistance mechanisms. Following a test cross that established that this mutation (furR3I) had indeed increased resistance, we made additional fur gene mutations and moved them to the H. pylori chromosome by PCR and transformation methods, scored them for effects on Fur protein function using a sensitive and efficient assay for changes in Mtx resistance levels, and interpreted their effects in terms of the recently determined structure of zinc-loaded H. pylori Fur protein [9]. Prominent among the mutations that increased resistance were seven that affected residues at the interface between Fur protein’s N-terminal DNA binding and C-terminal dimerization domains (furC40D, furT41I, Q1P, Q1K, M and Q and Q and E95G), and that might affect Fur protein’s iron-binding, dimer formation or stability, or Apo-Fur or Fe-Fur binding to cognate DNA sites; another resistance mutation altered a DNA binding surface (furK57T); and two others also affected Fur’s N terminal arm (furR5K and furE8K) (Fig. 2). Two other fur mutations were found recently by others [19] in Mtx resistant clinical isolates (furC70Y, furP14H). These mutations seemed to diminish Apo-Fur protein’s binding to the sodB promoter and thereby to allow increased superoxide dismutase synthesis and detoxification of Mtx’s activation products [19].

The ability of Fur to control transcription of many genes when iron-bound and other genes when iron-free is an important feature of this global regulatory protein. Using a PCR-targeted mutation and transformation strategy we randomly changed the codons for five residues that were implicated by structural considerations in iron binding (H42, E90, H97, H99 and E110; Fig. 2). Only nine binding pocket mutations were recovered without other inactivating changes in fur among the 62 transformants screened; each such mutation conferred less resistance than did a A fur allele (Fig. 4C); and two of them (furH42L and furH42V) caused slow growth. Most other transformants contained additional frameshift or scrambled sequence mutations, implying that most iron binding pocket changes were deleterious or lethal. A strain with one of these putatively lethal mutations (furH99R) was readily obtained if the gene also contained a DNA binding site mutation (furT65N). Accordingly, we suggest that the apparently lethal or deleterious effects of many mutations and the Mtx sensitization caused by those that were recovered reflect the same phenomenon: disruptions in the normally balanced expression of many Fur-regulated genes that vary in severity, and that in each case stem from decreased iron-dependent regulation without sufficient change in iron-independent (Apo) regulation. Just how iron
governs Fur protein’s conformation and activity is not yet known, but might entail changes caused by iron binding per se [33], by oxidation of bound iron [34], or by iron-catalyzed protein (histidine) oxidation [35].

Noteworthy in the context of the iron binding pocket point mutations, Mtz resistance was also decreased by nonsense mutations at codons 93, 99 and 115 in the 150 codon fur gene. The resultant truncated Fur proteins should retain the N terminal DNA binding domain, but lack the C terminal dimerization domain and a well-structured iron-binding pocket. We suggest that these remnants would retain Apo-Fur activity; and that their binding activity, although weakened by the inability to dimerize, nevertheless should be sufficient to impact on transcription of genes affecting Mtz susceptibility. Nonsense codons inserted closer to fur’s 5′ end exhibited the mild decrease in Mtz resistance characteristic of simple null (deletion) alleles, which would be explained if all Fur function is lost in these shorter remnants, and/or if remnant proteins are degraded.

**H. pylori** Fur’s distinctive N-terminal arm was identified as part of a well-defined structure in the recently released zinc-loaded Fur protein structure [9], with residue R3 participating in a hydrogen bond network with residues in the DNA-binding domain close to metal-binding residue H42 (Fig. 2). Our fur codon 3 mutagenesis results suggest, however, that this is not R3’s only significant interaction – if R3 were important only for stabilizing N-terminal arm-DNA binding domain interactions, all codon 3 mutations that altered Mtz resistance should have changed this phenotype in the same direction. However, two codon 3 mutations increased resistance (furR3I, furR3K), whereas two others markedly decreased resistance (furR3S, furR3N) more than did a simple Δfur (null) mutation. Mtz resistance was even further diminished when the Mtz-sensitizing alleles furR3S or furR3D were combined with furH99* (stop) (Fig. 2, 4A,D), which indicates that neither an ability to bind iron nor dimerization is needed for codon 3 mutations to affect Fur protein activity. We propose that the N terminal arm’s role in Apo-Fur is distinct from that in Fe-Fur. Precedents from similar functional studies on the heme-binding protein DurC of *B. subtilis* suggest that the N-terminal arm-DNA interaction in DurC may be sufficient to maintain DNA-binding activity, although weakened by the inability to dimerize [36].

**Materials and Methods**

**Fur protein structure modeling**

Molecular graphics images (Fig. 2) were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (http://www.cgl.ucsf.edu/chimera/docs/credits.html; supported by NIH P41 RR001081). [10] using recently reported *H. pylori* Fur structural data [9].

**H. pylori** strains and general methods

Most experiments reported here were carried out with Mtz resistant *H. pylori* strain M1.5. This strain had been derived from reference strain 26695 [40] by five sequential cycles of selection for increased resistance, each cycle associated with a new mutation that likely diminished the ability of cells to chemically reduce Mtz from prodrug to bactericidal agent (genes involved were *rdxA, f1xa, mdaB, ribF* and *fur*) [20]. Also used were strain 26695 wild type, its *rdxA f1xa* mutant derivative M2.2, and equivalent *rdxA f1xa* mutant derivatives of unrelated strains SS1, X47 and G27 [41–43]. *H. pylori* strains were cultured at 37°C in a standard microaerobic atmosphere (5% O2, 10% CO2, 85% N2) on brain heart infusion-7% horse blood agar plates with 0.4% Isovital and antibiotics amphotericin B (8 mg/l), trimethoprim (5 mg/l), and vancomycin (6 mg/l), chloramphenicol (15 mg/l) or kanamycin (25 mg/l) were added as needed to select for transformants. Metronidazole (Mtz) was added at various concentrations for quantitative scoring of susceptibility and resistance at concentrations appropriate for the strains being tested, as described [20,29]. Natural transformation was carried out by adding 7 μl of purified PCR product or 1 μg of genomic DNA to a lawn of cells growing exponentially on nonselective medium, and restreaking the population on selective (generally chloramphenicol containing) medium after 6–8 hrs or overnight incubation to obtain transformant colonies.

**PCR-based construction of strains with random mutations in fur**

Error prone PCR was carried out in 100 μl volumes containing 20 fmol of genomic DNA from a *fur-cat* (Fig. 3) derivative of strain 26695, 30 pmole of each primer (S5K, x4; Table S1), 5 unit of Taq polymerase, 0.2 mM of dGTP, dATP, 1 mM of dCTP, dTTP, 0.5 mM of MnCl2 in PCR buffer (1x buffer; 7 mM MgCl2, 50 mM KCl, 10 mM Tris-Cl (pH 8.3)). The PCR product was used to transform 26695 *Afur-aphA*, and chloramphenicol resistant (*Cam*) transformants were selected, sometimes with accompany-
incubated for 3 days. Then bacterial growth was restreaked on

Determination of Mtz resistance phenotypes

PCR-based construction of strains with targeted fur mutations

Alleles with mutations targeted to specific sites were constructed by assembling individual PCR products without need for recombinant DNA plasmid cloning. In brief, assembly depends by assembling individual PCR products without need for

Supporting Information

Table S1 Primer sequences used for *H. pylori* fur gene manipulation and analysis.

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<td>Conceived and designed the experiments: SSC PTC DEB. Performed the experiments: SSC. Analyzed the data: SSC PTC DEB. Contributed reagents/materials/analysis tools: SSC PTC DEB. Wrote the paper: SSC PTC DEB.</td>
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