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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 diseases 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 operator 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* cells [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 determined [9,11,14–17]. 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 amino acid long segment with no counterpart in Fur proteins and that affects DNA binding specificity [25]. Accordingly, we documented in early studies of phage Fur’s N-terminal arm, a functional. **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. doi:10.1371/journal.pone.0018236.g001

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 *sodB*, *frxA*, *rifB* and *mdaB*) [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). 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* (Δfur-aphA) allele), or simply deleted in PCR products containing a downstream *cat* gene (Δfur-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 *fur* decreases resistance whereas *furR3I* increases resistance, relative to *fur-set*, further emphasizes that *H. pylori* Fur protein’s N-terminal arm is functional.
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.

![Figure 2. Mutational changes in H. pylori Fur that affect Mtz resistance in distinct regions of the protein.](image)

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 along Fur family members.

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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 all 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

Figure 3. Strategy for efficient mutational analysis of H. pylori Fur. A) Transformation based strategy for placing any gene or mutation of interest at a predetermined place in the H. pylori chromosome. Recipient strains used here contain an aphA (amino phosphotransferase; kanamycin resistance) gene in place of fur. Homologies of at least ~0.5 kb between transforming DNA and the recipient chromosome both upstream of fur and downstream of the inserted cat (chloramphenicol acetyl transferase, resistance) gene allow efficient recombination and recovery of fur alleles by selection for chloramphenicol resistance. B) Two step strategy for directed sequence change within fur. Primers 2 and 3 overlap and cover the site to be changed; primers 1 and 4 lie upstream and downstream, respectively, of the fur-cat gene pair. Initial PCRs with primers 1 and 2, and separately with primers 3 and 4, generate two PCR products that overlap to an extent determined by the sequences of primers 2 and 3 (typically ~40 bp in our studies) and that contain the intended mutant allele(s). A second PCR with the two products of the first PCRs and primers 1 and 4 generates a product suitable for transformation into a Δfur recipient, as in panel A. An equivalent strategy was used to generate more complex multi-mutant fur alleles by splicing complementary singly mutant segments of fur together; and also to generate a deletion with defined endpoints such as furD2–7, which was made with long primers whose 5’ and 3’ portions corresponded to sequences flanking the segment to be deleted. The sequences of primers used for PCR amplification, mutation and sequencing are listed in Table S1.

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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-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 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 M1.5 fur-wt. 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 (fur2–7 in Fig. 4A) was made similarly. It conferred a Mtz resistance phenotype equivalent to that of the total fur gene deletion (fur-aphA) (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

Figure 4. Graphic presentation of effects of representative fur alleles on Mtz susceptibility. The fur substitution mutations indicated here are named using the one letter code according to amino acid in wild type, its numerical position in the H. pylori Fur protein, and the amino acid encoded by the mutant allele. For example, furR3I indicates a mutation at codon 3 that changes arginine to isoleucine at Fur position 3. The horizontal scales represent the highest level of Mtz in μg per ml in agar medium (0 to 280) that allows 100% efficiency of colony formation by cells of a given derivative of strain M1.5 (mutant in rdxA, frxA, ribB, mdaB), defined by its fur allele. Thus, the furR3I strain forms colonies with 100% efficiency on medium with 230 μg Mtz/ml, and with less than 1% efficiency on medium with 250 μg Mtz/ml (phenotype designated 230R,250S in text). Mtz concentrations in media used for these analyses were 0, 3, 5, 8, 16, 32, 64, 50, 90, 130, 160, 190, 220, 230, 250, 260 and 280 μg/ml. A) Effects on Mtz resistance of sequence changes in H. pylori Fur’s N terminal arm shows that this arm is important functionally. B) Point mutations that increased resistance obtained by random PCR-based mutagenesis of the entire fur gene, transformation as in Fig. 3, and initial selection for colony formation on medium with chloramphenicol and 200 μg Mtz per ml. C) Effects of changes in residues likely to be part of the iron binding pocket. The majority of transformants containing changes at these positions also contained inactivating (generally frameshift) mutations elsewhere in the fur gene, suggesting that most such putative iron binding pocket changes were deleterious or lethal. The subset that were recovered without additional changes (depicted here) are thus likely to be leaky alleles, possibly still able to bind iron with low efficiency. D) Decrease in Mtz resistance caused by H. pylori Fur truncation at positions indicated by asterisks (nonsense (stop) codon mutations). Especially noteworthy are the extreme Mtz sensitizations caused by several double mutant alleles, in particular furR3T,H99* and furR3D,H99*, which are far more severe than the sensitizations caused by their component single mutant alleles. Not depicted in the Figure were also several dramatic Mtz sensitization phenotypes conferred by fur alleles with unintended frameshift mutations near codon 110 and that had emerged from an attempt to change E110. Phenotypes of 64R, 32R, and 32S were obtained in M1.5 derivatives whose altered C terminal amino acid sequences from position 109 were IRFCRP*, IQFARP* and HSLCRP*, respectively (* designates stop codon). The sequence of the corresponding region of Fur wild type is IEFADPE.

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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 Fur-aplA, as described above. Four transformants selected only for chloramphenicol resistance marker. Tests of transformants verified that these transformants did indeed cause increased Mtz resistance by transforming chloramphenicol resistance marker. Tests of transformants verified contained single mutations affecting protein positions 40, 41, 57 and 95.

Since Mtz treatment is mutagenic [26] and these transformants had been selected on Mtz-containing agar, we tested if their fur alleles did indeed cause increased Mtz resistance by transforming them into strain M1.5 Fur-aplA, with selection only for the linked chloramphenicol resistance marker. Tests of transformants verified that each mutation had indeed increased Mtz resistance, relative to the fur-wt allele (Fig. 4B). Based on the Fur protein structure (Fig. 2) [9], changes at positions 40, 41 and 95 should affect the interface between the N terminal DNA binding and C terminal dimerization domains and/or the iron binding pocket's conformation or flexibility; the change at position 57 should affect a DNA binding interface.

**Iron binding pocket residues that affect Mtz resistance or viability**

Given that both the Fe- and Apo- forms of H. pylori Fur have regulatory activity [4,5] (Fig. 1), five residues that seemed likely to affect the conformation of the metal-binding pocket and/or make direct contact with bound iron (Fig. 2B) were targeted next for mutagenesis. We began by attempting to make a strain M1.5 derivative with furH99R, an allele that should be equivalent to the Salmonella furH90R allele that had resulted in iron-independent Fur-mediated regulation of acid resistance [31–39]. However, 10 of 12 Cam’ transformants of strain M1.5 Fur-aplA generated using PCR products made with H99R-specific primers (as in Fig. 3) contained frameshifts; one contained a scrambled sequence; and one contained the original wild type sequence. None contained only the desired H99R change. Similarly, each of three Cam’ transformants made with the same PCR products, but using as recipient a Fur-aplA derivative of wild type strain 26695 (the Mtz’ ancestor of strain M1.5; wild type alleles of genes rdxA, fexA, ribF, mduB; ref. 20) also contained a frameshift or scrambled sequence. Collectively, these data suggested that this furH99R allele is deleterious or lethal in H. pylori, independent of the status of four genes likely to affect cellular reductive potential.

In parallel experiments, we generated a furT65N allele, where residue Y65 in H. pylori Fur corresponds to the critical Y55 component of a DNA binding helix of E. coli Fur [32]. Both furT41A and fur-wt containing DNAs were used as PCR templates (Fig. 3B). The furT65N allele caused severe Mtz sensitization in strain M1.5, in both furT41-wild type and furT41A (resistance-enhancing) contexts: 64R,90S and 90R,130S phenotypes, respectively. This contrasts with the 190R,220S and 260R,20S phenotypes of isogenic strains containing a non-mutant furY65 site and the 160R,190S phenotype of the isogenic Afur strain (Fig. 4C). Having found furT65N to decrease Mtz resistance, we next generated furH99R,Y65N double and furH99R,T41A,Y65N triple mutant alleles. The triple mutant was made first, using a furY65N,T41A template and furH99R-containing primers (Fig. 3); 10 of 11 Cam’ transformants contained the desired triple mutations with no additional mutations in fur. The double mutant was made similarly, starting with triple mutant DNA as PCR template, and primers with wild type sequence covering codon 41; each of the two Cam’ transformants analyzed contained the desired furY65N,H99R (fur codon 41-wt) allele, with no additional mutations in fur. These results indicate that furH99R’s deleterious or lethal impact can be compensated by the furY65N mutation, probably because it diminishes Fur protein-DNA binding. 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 changes was carried out using pairs of mutagenic primers designed to generate random amino acid replacements of critical histidine and glutamic acid residues (H42, H97, H99, E90 and E110; Fig. 2), using fur-wt cat DNA as template and strain M1.5 Fur-aplA as the transformation recipient (Fig. 3). The fur genes of 62 transformants selected solely for chloramphenicol resistance were sequenced (average ~12/targeted site). Collectively, only nine amino acid replacement mutations were obtained at these various sites with no other unintended changes (Fig. 4C, below). Rather, most transformants contained additional changes, variously frameshifts (27/62), scrambled sequences (9/62) and stop codons (2/62), or were not mutant (11/62). This low yield of simple single mutations suggested that most changes affecting H. pylori Fur’s iron-binding pocket were deleterious or lethal, and thus recoverable in transformants only if the fur gene had been inactivated.

Each of the simple iron binding pocket mutations that we did obtain caused severe Mtz sensitization (ranging from 3R,8S to 64R,90S, depending on allele) (Fig. 4C). The two most extreme alleles, furH42V and furH42L, also caused slower growth on Mtz-free agar (colonies 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 these 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 furY65N and also furE90A or Q 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.

**Mtza 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...
survival of M2.2 on agar with 32

diminished by the

furT41A

resistance-associated mutations, recipient strains were prepared by

nitroreductase genes are responsible for the first two steps in

and aliquots of diluted cultures were spotted on complementary

This result indicates that the

ribF

contribute to Mtz resistance. To learn if the changes in resistance

epistatic'' to (masks effect of) several other mutations that

(position 86 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 furR3I,H99* and furR3D,H99* each

conferred a 16R,32S 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 circuity. 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

frxA

nitrreductase genes are responsible for the first two steps in
development of Mtx 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 fxaD (D Dailidiene and DE Berg, unpublished data).

This result indicates that the

rdxA-act (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

fur-aphA1

allele into the ancestral wild type strain

26695 and into its derivative mutant only in

rdxA

and fxaD ("strain M2.2’"); contains functional alleles of genes

ribF

and

mdaB.

These

fur

derivative strains were then transformed with DNA

containing informative cat-linked fur alleles.

Neither the

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-act being epistatic on other

resistance gene mutations. In strain M2.2 (null alleles in

rdxA

and

fxaD), 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,128). The

furR3S mutation also decreased resistance in the M2.2 background,

albeit rather subtly: like

fur-act,

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- and

furR3S alleles allowed survival of 10^{-7} and only 10^{-5}, 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 S81, X47

and G27, each with null alleles of

rdxA

and

frxA

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

fur

furR3S

and

furH99

alleles and enhanced by the

furR3I

and

furT41A

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

imbalance 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

furG40D

and

furH99

Q

and

furE95G,

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

furK577T;

and two others also

affected Fur’s N terminal arm

furR3K

and

furE8K

(Fig. 2). Two

other fur mutations were found recently by others [19] in

Mtx resistant clinical isolates

furC79Y, furP114S.

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 transfomants screened; each such

mutation conferred less resistance than did a

fur

allele (Fig. 4C); and two of them

furH42L and

furH42V

caused slow growth. Most

other transfomants contained additional frameshift or scrambled

sequence mutations, implying that most iron binding pocket

changes were deleterious or lethal. A strain with one of these

sequence mutations, implying that most iron binding pocket

alleles contained a DNA binding site mutation

furH99

and

furR3I alleles, and that the regulatory

imbalance 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.
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 mutation. Mtz resistance was even further diminished when the furR3I, furR3K, furR3S, furR3N 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 other regulatory proteins [21–25] suggest that the Fur arm might affect Fur regulon gene expression via interaction with target DNAs and/or a cellular effector such as RNA polymerase.

The need for further analysis of just how specific changes in H. pylori’s Fur protein affect its various actions and associated phenotypes is also emphasized by a recent study of alanine replacement mutations affecting iron binding pocket residues [18]. The growth of furE90A-containing H. pylori under iron-replete conditions depressed transcription of the Apo-Fur-repressed iron storage gene pfr, whereas transcription of an Apo-Fur-induced gene, amiE, was not much elevated, nor did the furE90A mutation significantly affect iron-loading of Fur protein in vitro. In addition, a furE110A allele, which one might have expected to affect iron-binding similarly had effects distinct from those of furE90A on pfr and amiE gene transcription in vivo and on iron loading and protein dimerization in vitro [18]. These divergent results are in accord with findings of distinct functional roles for residues at different positions in the metal binding pocket of another regulatory metalloprotein [36].

The sensitivity and convenience of Mtz resistance as a diagnostic phenotype and the metabolic changes that particular H. pylori Fur alleles elicit, should encourage further studies. We envision (i) further mutant hunts, using other informative phenotypes such as susceptibility to low pH or oxidative stress and expression profiling, expecting that many of the point mutations obtained will have far stronger effects than those conferred by standard gene inactivation; (ii) analyses of mutational effects on Fur activity, transcript profiles, binding to cognate DNA regulatory sites and protein interaction partners, and the structures involved [6,13,18,31–37]; (iii) selection for and analysis of compensatory or suppressor mutations within fur or elsewhere in the genome, starting with alleles such as furR3N or furH99* that decrease resistance [22,23,30]; and (iv) placement of fur under control of a separately regulated promoter [39], to allow studies of the expression of diverse Fur regulon genes, uncomplicated by Fur regulation of its own expression, and to allow potentially lethal alleles to be recovered efficiently, the immediate consequences of their expression examined, and suppressors of their lethality obtained and characterized.

In conclusion, the simple phenotypic results obtained in our fur mutation studies to date, and prospects for informative future analyses illustrate the power of PCR- and chromosomal transformation based mutational analysis of protein structure and function and regulatory circuitry. This strategy should be applicable to many genes of interest in any transformable microbial species.

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, fxa, mdaB, ribF and fur) [20]. Also used were strain 26695 wild type, its rdxA fxa mutant derivative M2.2, and equivalent rdxA fxa 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% isovitalex 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 (x5K, 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-
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 on overlaps of $\geq 20$ bp at the ends of DNAs to be joined together, which, in turn, result from the design of PCR primers used in amplification [27,28]. To construct several different mutant alleles simultaneously, a forward primer upstream of fur (#1 in Fig. 3B; x3K in Table S1) and a reverse mutagenic primer (#2 in Fig. 3B) was used to amplify part of fur, and a forward mutagenic primer (#3 in Fig. 3B) and a reverse primer downstream of cat (#4 in Fig. 3B; x4 in Table S1) was used to generate a complementary part of fur and the downstream cat gene and adjacent $H. pylori$ sequences. A second round of PCR assembly using mixture of these two fragments and primers 1 and 4 yielded a full-length fur,cat product likely to be mutant at the site dictated by mutagenic primers #2 and #3. This product was used in transformation (Fig. 3).

Determination of Mtz resistance phenotypes

Frozen $H. pylori$ cultures were streaked onto Mtz-free BHI agar and incubated for 3 days. Then bacterial growth was restreaked on fresh Mtz-free BHI agar and incubated for 1 day. The resulting young exponentially growing cells were suspended in phosphate buffered saline; a series of 10-fold dilutions of these suspensions was prepared; and $10 \mu l$ of each dilution was spotted on one half of freshly prepared BHI agar plates containing appropriate concentrations of Mtz. A strain was considered to be susceptible to concentrations of Mtz that decreased its efficiency of colony formation by single cells at least 10 fold. Each such strain was tested at least twice to assess reproducibility, generally along with a different strain that might differ slightly in resistance, and that was spotted in parallel on the other half of the same plate. This protocol for quantitative and reproducible determination of levels of Mtz susceptibility or resistance, uncomplicated by Mtz's protocol for quantitative and reproducible determination of levels of Mtz susceptibility or resistance, uncomplicated by Mtz's

**Supporting Information**

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

**Author Contributions**

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.


