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H. pylori apo-Fur Regulation Appears Unconserved Across Species

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Abstract

The Ferric Uptake Regulator (Fur) is a transcriptional regulator that is conserved across a broad number of bacterial species and has been shown to regulate expression of iron uptake and storage genes. Additionally, Fur has been shown to be an important colonization factor of the gastric pathogen *Helicobacter pylori*. In *H. pylori*, Fur-dependent regulation appears to be unique in that Fur is able to act as a transcriptional repressor when bound to iron as well as in its iron free (*apo*) form. To date, *apo*-regulation has not been identified in any other bacterium. To determine whether Fur from other species has the capacity for *apo*-regulation, we investigated the ability of Fur from *Escherichia coli*, *Campylobacter jejuni*, *Desulfovibrio vulgaris* Hildenborough, *Pseudomonas aeruginosa*, and *Vibrio cholerae* to complement both iron-bound and *apo*-Fur regulation within the context of an *H. pylori fur* mutant. We found that while some Fur species (*E. coli*, *C. jejuni* and *V. cholerae*) complemented iron-bound regulation, *apo*-regulation was unable to be complemented by any of the examined species. These data suggest that despite the conservation among bacterial Fur proteins, *H. pylori* Fur contains unique structure/function features that make it novel in comparison to Fur from other species.

Introduction

Helicobacter pylori persistently colonizes the gastric mucosa of the majority of the world's human population (Blaser 1998). This fact seems remarkable when one considers that this site encounters large fluctuations in pH (McArthur and Feldman 1989), iron availability (Andrews *et al.* 2003), and other stresses (Seyler *et al.* 2001). Thus, in order to survive in this niche, *H. pylori* must be able to adapt to this dynamic, tumultuous environment. Indeed, a number of regulatory proteins in this organism have been shown to serve as essential components required for adaptation to stressful environments (Bury-Mone *et al.* 2004; Delany *et al.* 2005; Gancz *et al.* 2006). Included among these is the Ferric Uptake Regulator (Fur), which is involved in *H. pylori* colonization (Bury-Mone *et al.* 2004; Gancz *et al.* 2006) and is a necessary component for adaptation to low pH (Bijlsma *et al.* 2002) and iron limitation (Bijlsma *et al.* 2000).

In most organisms, iron is essential (Ratledge and Dover 2000) because it plays a role in respiration, electron transport, and is a required cofactor for many enzymes. Paradoxically, too much iron is as detrimental as insufficient amounts of iron since excess free iron leads to the Fenton reaction, which results in the formation of DNA-damaging and protein denaturing hydroxyl radicals (Gutteridge *et al.* 2001). Thus, there must be a delicate balance between acquiring a sufficient amount of iron but not so much as to overload the system.

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Indeed, this balance is achieved in many Gram positive and Gram negative bacterial species by intricate control over the transcription of iron uptake and storage genes by Fur.

Classically, Fur functions as a transcriptional repressor protein that binds to conserved promoter regulatory sequences known as Fur boxes (Hantke 2001). These Fur boxes often overlap the −10 and −35 promoter elements. Thus when iron is available, Fur binds its ferrous iron cofactor, dimerizes and binds to the Fur box. This complex prevents the binding of the RNA polymerase and gene expression is repressed. Conversely, as iron becomes limited, there is an insufficient amount of the ferrous cofactor to bind to Fur and thus, the protein is unable to dimerize and bind to the promoter elements. This allows RNA polymerase to bind and the gene is transcribed. *H. pylori* uses this type of classical regulation to control expression of several genes including the aliphatic amidase, *amiE*, which plays an important role in ammonia production through the hydrolysis of aliphatic amides (Ernst *et al.* 2005; Gancz *et al.* 2006; van Vliet *et al.* 2003). However, Fur regulation in *H. pylori* is more complex than this classic model since Fur has also been shown to repress expression of some additional promoters in an iron depleted (*apo*) form (Delany *et al.* 2001; Delany *et al.* 2001; Ernst *et al.* 2005). For this *apo*-regulation, in the absence of iron the *apo*-Fur protein can bind to the promoters of its target genes and block transcription. Thus, genes repressed by *apo*-Fur are transcribed in iron-replete conditions. Currently, the *apo*-Fur regulon is predicted to contain 16 gene targets (Ernst *et al.* 2005; Gancz *et al.* 2006). Of these targets, only *sodB*, a superoxide dismutase important for oxidative defense, and *pfr*, an iron storage molecule, have been definitively shown to be directly regulated by *apo*-Fur (Spiegelhalder *et al.* 1993; Delany *et al.* 2001; Ernst *et al.* 2005). Expression of both of these genes is repressed by *apo*-Fur when iron is limited, but this repression is lost in a *fur* mutant strain.

Recent microarray analyses of *Campylobacter jejuni* (Holmes *et al.* 2005) and *Desulfovibrio vulgaris* Hildenborough (Bender *et al.* 2007) suggest that *apo*-Fur regulation may occur in these organisms; however, direct binding of *apo*-Fur to any identified target genes has not been shown in these organisms. Indeed, despite the fact that Fur has been extensively studied in many other organisms (Carpenter *et al.* 2009) there is currently no direct evidence that bacterial species other than *H. pylori* utilize *apo*-Fur regulation. This fact suggests that *H. pylori* Fur contains unique structure/function features in comparison to Fur from other bacterial species. Alternatively, it is possible that Fur from other bacterial species encodes the capacity for *apo*-regulation, but this form of regulation simply has not been identified in these organisms. To begin to examine these possibilities, herein we describe studies that investigate the ability of Fur from other bacterial species to complement both iron-bound and *apo*-Fur regulation within the context of an *H. pylori fur* mutant.

Materials and Methods

Bacterial strains and growth

The strains and plasmids used in this study are listed in Table 1. *H. pylori* strains were maintained as frozen stocks at −80° C in brain heart infusion medium supplemented with 20% glycerol and 10% fetal bovine serum (FBS). Bacteria were grown on horse blood agar plates containing 4% Columbia agar base (Neogen Corporation, USA), 5% defibrinated horse blood (HemoStat Labs, USA), 0.2% β-cyclodextrin, 10 μg/ml vancomycin (Sigma, USA), 5 μg/ml cefsulodin (Sigma, USA), 2.5 U/ml polymyxin B (Sigma, USA), 5 μg/ml trimethoprim (Sigma, USA), and 8 μg/ml of amphotericin B (Amresco, USA). As noted in Table 1, cultures and plates were supplemented with 8 μg/ml chloramphenicol (Cm) (EMD Chemicals Inc., USA), and/or 25 ug/ml kanamycin (Kan) (Gibco, USA). All *H. pylori* was grown in gas evacuation jars under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) generated by Anoxomat gas evacuation (Spiral Biotech, USA).

Construction of heterologous Fur expression strains

Translational fusions in which the *H. pylori fur* promoter and 5′ nontranslated region, up to but not including the *H. pylori* Fur start codon, was directly fused to the start codon of the *fur* coding sequence of *C. jejuni* 11168 (Parkhill *et al.* 2000), *D. vulgaris* Hildenborough NCIMB 8303 (Heidelberg *et al.* 2004), *E. coli* O157::H7 EDL933 (Perna *et al.* 2001), *P. aeruginosa* PAO1 (Stover *et al.* 2000) or *V. cholerae* N16961 (Heidelberg *et al.* 2000) were constructed. In designing the translational fusions, the native *H. pylori* promoter and Ribosomal Binding Site (RBS) were used to bypass any potential problems with altered expression of a foreign *fur* promoter or RBS in the *H. pylori* system. For each construct, we utilized Splicing by Overlap Extension (SOE) PCR (Horton *et al.* 1993) to fuse the *H. pylori* promoter sequence to the heterologous Fur coding sequences. This was accomplished in a series of three PCR reactions using the primers listed in Table 2.

Briefly, template DNA from *H. pylori* G27 was isolated using the Invitrogen Easy DNA kit (USA), and used in combination with genomic DNA from *C. jejuni* 11168 (provided by D. Hendrixson), *D. vulgaris* Hildenborough NCIMB 8303 (provided by J. Wall), *E. coli* O157::H7 EDL933 (provided by A. O'Brien and L. Teele), *P. aeruginosa* PAO1 (provided by V. Lee), or *V. cholerae* N16961 (provided by A. Camilli). In the first and second PCR reactions, the *H. pylori fur* promoter was amplified such that the 3′ end of the fragment contained a complementary and overlapping region with the individual heterologous *fur* sequences and the heterologous *fur* coding sequences were amplified with a 5′ complementary overlapping extension for the *H. pylori fur* promoter sequence, respectively. In the final reaction, each of these products was mixed together, the complementary regions annealed and the fused product amplified using the extreme flanking primers (Table 2). Each of these *H. pylori fur* promoter – heterologous *fur* coding sequence products was initially subcloned into the pGEMT-Easy vector (Promega, USA) (Table 1) prior to digestion and ligation into the appropriately digested pTM117 vector, which has previously been shown to be an efficient complementation vector for *fur* in *H. pylori* (Carpenter *et al.* 2007). In addition, DSM343, a strain carrying a pTM117 vector carrying the *H. pylori fur* promoter driving expression of *H. pylori fur* (pDSM340) was prepared for use as a positive control (Carpenter *et al.* 2007). Each of these vectors was next transformed into DSM300, which is a *H. pylori ΔfurH. pylori* mutant of strain G27 (Gancz *et al.* 2006). Transformants were selected on the appropriate antibiotics (Table 1). To verify that each of the individual heterologous fusions was correct and contained no mutations, each of the pTM117 vectors (pDSM515, pDSM526, pDSM560, pDSM652, pDSM758) was subsequently recovered from each of the *H. pylori* transformant strains and sequenced.

RNase Protection Assays

Each of the heterologous expression strains, as well as the wild type and *ΔfurH. pylori* controls, were grown for 18 hours in liquid culture (Brucella broth (BB) supplemented with 10% FBS, 50 μg/ml vancomycin, and 25 μg/ml Kan to ensure maintenance of the plasmid). One half of each culture was removed for RNA extraction (t_0) while the other half was depleted of iron by the addition of 200 μM of the iron chelator 2,2′ dipyridyl (dpp). After one hour of chelation (t_{60}) these cells were then harvested for RNA extraction. RNA was extracted as previously described (Thompson *et al.* 2003). To examine expression of the *fur* transcript from the plasmid, riboprobe templates were constructed for *C. jejuni*, *E. coli*, *D. vulgaris*, *H. pylori*, *P. aeruginosa*, and *V. cholerae fur* using the primer pairs listed in Table 2. To measure iron-bound and *apo*-Fur regulation, riboprobe templates were also generated using the primer pairs listed in Table 2 for *amiE* and *pfr*, respectively. The resulting *fur*, *amiE* and *pfr* amplicons were ligated to pGEMT-easy (Promega, USA) and riboprobes were generated with the Maxiscript kit (Applied Biosystems, USA) and 50 μCi [^{32}P] UTP (Perkin Elmer, USA). 1.5 μg of total RNA was then used to conduct RNase protection assays

(RPAs) with the RPA III kit (Applied Biosystems, USA) as previously described (Carpenter *et al.* 2007). The gels were exposed to phosphor screens. The screens were scanned using a FLA-5100 scanner (Fujifilm, USA) and the intensity of protected bands was quantified with Multi-Gauge software (version 3.0, Fujifilm, USA).

Western blotting

To confirm expression of each Fur species, bacteriallysates were prepared from the heterologous strains grown as described above. Protein concentration was measured using the BCA protein assay (Thermo Scientific, USA) and equal concentrations of each sample were separated by sodium dodecyl sulfate-polyacrylamidegel electrophoresis (SDS-PAGE) using an 18% separating gel. The separated proteins were transferred to nitrocellulose membranes using a semidry transfer apparatus (Owl; Thermo Scientific, USA), and membranes were probed with anti-Fur antibodies. Given that antibodies specific for each of the individual Fur species were not available, we utilized polyclonal antibodies from available species and relied on the conservation of the protein to aid in the detection of Fur. Membranes were first probed with a 1:100 dilution of *P. aeruginosa* Martha 2472 polyclonal rabbit anti-Fur antibody (a kind gift from M. Vasil), followed by a 1:20,000 dilution of HRP-conjugated bovine anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). Proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific/Pierce, USA) and a LAS-3000 Intelligent Dark Box with LAS-3000 Lite capture software (Fujifilm, USA).

In order to detect the other heterologous Fur proteins, the membrane was then stripped by incubation at approximately 50°C in stripping solution (2% SDS, 62.5 mM Tris HCl pH 6.8, 10mM DTT) for 30 minutes, and reprobed with a 1:1,000 dilution of rabbit polyclonal anti-*C. jejuni* Fur antibody (a kind gift from A. Stintzi) followed by a 1:20,000 dilution of HRP-conjugated bovine anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, USA). After detection and scanning, the membrane was then stripped again and probed with a 1:100 dilution of rabbit polyclonal anti-*H. pylori* Fur antibody, which was prepared using the Rabbit Quick Draw protocol and produced by Pocono Rabbit Farm and Laboratory (Carpenter *et al*. 2010 Submitted). This was followed by a 1:20,000 dilution of HRP-conjugated bovine anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, USA) and detected as described above.

Results

Sequence conservation among Fur species

Comparison of the amino acid sequence of Fur from *H. pylori* to Fur encoded by several bacterial species in which Fur has been well studied (Pohl *et al.* 2003; Holmes *et al.* 2005; Bender *et al.* 2007; Sheikh and Taylor 2009) showed a moderate degree of conservation among the Fur proteins (Table 3, Fig. 1). Of note, of the species examined, the highest degrees of identity were found with *C. jejuni*, which is a close relative of *H. pylori*, and *D. vulgaris*, which is more distantly related to *H. pylori*. Together these two microbes remain the only other species that have been suggested to utilize *apo*-Fur regulation (Holmes *et al.* 2005; Bender *et al.* 2007). However, moderate levels of identity and similarity were also found in comparison to *E. coli*, *P. aeruginosa*, and *V. cholerae* Fur, none of which are currently suspected to utilize *apo*-Fur regulation. Based on this conservation, we wondered if any of these heterologous Fur species would be able to complement classic iron-bound and/or *apo*-Fur regulation when expressed within the context of a *H. pylori fur* mutant.

Analysis of iron-bound Fur complementation

To determine whether the individual heterologous Fur constructs could complement iron-bound Fur regulation in the *Δfur* G27 strain, changes in the transcription of *amiE* were monitored in response to iron availability; *amiE*, encodes an aliphatic amidase and is known to be repressed by iron-bound Fur (van Vliet *et al.* 2003). As shown in Figure 2A, addition of the iron chelator, dpp, to the wild type strain resulted in a large increase in *amiE* expression (4.9 fold). However, this increase is lost in the *Δfur* strain (0.6 fold), which additionally shows increased basal level expression of *amiE* even in the presence of iron (Fig. 2A). These results are in accordance with *amiE* being repressed by the iron bound form of Fur; in the absence of iron, iron-free Fur is no longer able to bind to the Fur box and repress expression of *amiE*. For each of the heterologous strains, three to four biological repeats of the chelation and RPAs were repeated and the fold change relative to t_0 calculated. In order to show the reproducibility of the RPA data, the data is represented in a graphical format in Figures 2B and 2C. In these graphs, the fold change for each strain and biological repeat is displayed as a point on the graph. Additionally, to allow for easy comparison between the strains, the median fold change is depicted as a bar. As expected (Carpenter *et al.* 2007), increased *amiE* expression in response to iron chelation was partially restored (2.6 fold) in the strain expressing G27 Fur in the context of the complementation vector pTM117 (Fig. 2B). Analysis of *amiE* expression in the strains carrying the heterologous Fur constructs showed the following changes: *C. jejuni* (1.4 fold), *D. vulgaris* (1.2 fold), *E. coli* (2.0 fold), *P. aeruginosa* (0.7 fold) and *V. cholerae* (1.4 fold).

Given that the *Δfur* strain showed an increased basal level expression of *amiE* (3.6 fold) even in the presence of iron (Fig. 2A), we also assessed whether there was a difference in the relative level of expression of *amiE* between strains at the t_0 time point since this also would be an indication of complementation. For this analysis, the level of *amiE* for each of the heterologous constructs at t_0 was calculated relative to the level expressed in the wild type at t_0. As expected (Carpenter *et al.* 2007), basal level expression of *amiE* in the strain expressing G27 Fur in the context of pTM117 was similar to wild type (1.2 fold), thus indicating that Fur carried on this vector is able to complement a chromosomal *fur* mutation (Fig. 2C). Analysis of *amiE* basal level expression in the strains carrying the heterologous Fur constructs showed the following changes: *C. jejuni* (1.5 fold), *D. vulgaris* (3.0 fold), *E. coli* (1.1 fold), *P. aeruginosa* (3.5 fold), and *V. cholerae* (1.8 fold). Taken together with the above comparison, these data indicate that classic iron-bound Fur regulation in *H. pylori* is able to be partially complemented by Fur from *C. jejuni*, *E. coli*, and *V. cholerae* but not by *D. vulgaris* or *P. aeruginosa* Fur.

Comparison of *apo*-Fur complementation

Despite the identity and similarity among the Fur proteins (Table 3, Fig. 1), *apo*-Fur regulation has thus far only been definitively identified in *H. pylori* (Spiegelhalder *et al.* 1993; Delany *et al.* 2001; Ernst *et al.* 2005). To determine whether the individual heterologous Fur proteins could complement *apo*-Fur regulation in the *Δfur* G27 strain, changes in the transcription of *pfr* were monitored in response to iron availability; *pfr*, encodes a prokaryotic nonheme iron-containing ferritin that is repressed by *apo*-Fur (Delany *et al.* 2001). As shown in Figure 3A, addition of dpp to the wild type strain resulted in a large decrease in *pfr* expression (10.0 fold). However, this decrease is lost in the *Δfur* strain (1.1 fold). These results are in accordance with *pfr* being repressed by the *apo*-Fur (Delany *et al.* 2001; Ernst *et al.* 2005); in the absence of iron, *apo*-Fur binds to the Fur box and represses expression of *pfr*. Once again, for each of the heterologous strains, three to four biological repeats of the chelation and RPAs were performed and the fold change relative to t_0 calculated. As expected (Carpenter *et al.* 2007), decreased *pfr* expression in response to iron chelation was partially restored (3.0 fold) in the strain expressing G27 Fur in the

context of pTM117 (Fig. 3B). Analysis of *pfr* expression in the strains carrying the heterologous Fur constructs showed the following changes: *C. jejuni* (1.2 fold), *D. vulgaris* (0.9 fold), *E. coli* (1.1 fold), *P. aeruginosa* (1.3 fold), and *V. cholerae* (1.1 fold).

Given that the *Δfur* strain showed an increased level of expression of *pfr* (8.6 fold) in the absence of iron (Fig. 3A), we additionally asked whether there was a difference in the relative level of expression of *pfr* between strains at the t_{60} time point, since this would also be an indication of complementation. For this analysis, the level of *pfr* for each of the heterologous constructs at t_{60} was calculated relative to the level expressed in the wild type at t_{60}. As expected (Carpenter *et al.* 2007), basal level expression of *pfr* in the strain expressing G27 Fur in the context of pTM117 was similar to wild type (1.0 fold), thus indicating that Fur carried in the context of pTM117 is able to complement a chromosomal *fur* mutation (Fig. 3C). Analysis of *pfr* basal level expression in the absence of iron in strains carrying the heterologous Fur constructs showed the following changes (Fig. 3C): *C. jejuni* (10.0 fold), *D. vulgaris* (7.8 fold), *E. coli* (11.9 fold), *P. aeruginosa* (14.5 fold) and *V. cholerae* (16.0 fold). Thus, all of the heterologous fusions exhibited a *Δfur* phenotype for *apo*-Fur regulation. Taken together with the above comparison, these data indicate that *apo*-Fur regulation in *H. pylori* is unable to be complemented by Fur from *C. jejuni*, *D. vulgaris*, *E. coli*, *P. aeruginosa*, or *V. cholerae*. This may suggest that *apo*-Fur regulation depends on unique structural features of *H. pylori* Fur that are absent in the other Fur proteins.

Confirmation of expression and translation of *fur* transcript

Since iron-bound complementation was not observed for all of the heterologous constructs and *apo*-Fur complementation was only observed in the control Δ*fur* strain expressing *H. pylori* Fur on pTM117, we next confirmed that these results were not biased by an inability of the heterologous *fur* to be transcribed or for transcript to be stably maintained in *H. pylori*. To address these concerns, a riboprobe specific for each heterologous Fur species was generated using the primer pairs indicated in Table 2, and RPAs were conducted to detect each *fur* transcript. *fur* expression was detected in each strain (data not shown) therefore, lack of gene expression or instability of the heterologous mRNA is not responsible for the lack of complementation of Fur regulation.

Finally, given that we could detect transcript for each heterologous Fur species, we asked whether or not we could also detect each of the Fur proteins. As shown in Figure 4A, the *P. aeruginosa* Fur antibody was able to detect *E. coli*, *P. aeruginosa*, and *V. cholerae* Fur expression within the context of the *H. pylori Δfur* strain. The *C. jejuni* Fur antibody was able to detect expression of *C. jejuni*, *E. coli*, *P. aeruginosa*, and *V. cholerae* Fur (Fig. 4B) and the *H. pylori* antibody was able to detect expression of the *H. pylori*, *C. jejuni*, *E. coli*, *D. vulgaris*, and *P. aeruginosa* Fur proteins (Fig. 4C). Taken together, these data indicate that each of the heterologous Fur species is translated and accumulated within the context of the *Δfur H. pylori* strain (Fig. 4). Furthermore, since each of the various polyclonal Fur antibodies were unable to detect all Fur species, these results imply that despite the identity and similarity among the proteins (Table 1), there must be considerable Fur structural differences among the various species.

Discussion

Fur has been characterized in a diverse number of bacterial species, and shown to play a crucial role in iron homeostasis (Ernst *et al.* 1978; Hantke 1984; Ochsner *et al.* 1995; Horsburgh *et al.* 2001; van Vliet *et al.* 2002; Fiorini *et al.* 2008). Typically, Fur only acts as a repressor when bound to iron. Despite extensive study, to date, *H. pylori* Fur holds the distinction of being the only Fur definitively shown to repress in the absence of its iron cofactor (Bereswill *et al.* 2000; Ernst *et al.* 2005). Though plasmid complementation

systems are often not as efficient as chromosomal borne systems, overall, our data indicate that both iron-bound and *apo*-Fur regulation can be partially complemented by *H. pylori* Fur carried on a plasmid vector and expressed in the *Δfur* strain (Fig. 2C, 2B). Additionally, iron-bound Fur regulation can be partially complemented by expression of *C. jejuni, E. coli,* and *V. cholerae* Fur in *H. pylori Δfur*. However, *apo*-Fur regulation is unable to be complemented by any of the examined Fur proteins from the five other bacterial species. This strongly suggests that *H. pylori* Fur contains unique structure/function features in comparison to Fur from other bacterial species. In turn, these features likely affect the ability of Fur to recognize and bind its DNA target. *H. pylori* is an A/T-rich organism (approximately 60%) (Alm *et al.* 1999; Baltrus *et al.* 2009), and the Fur box consensus sequence appears less conserved among the iron-bound Fur regulated *H. pylori* genes than the consensus sequences within these other organisms (Merrell *et al.* 2003). Indeed, previous studies have suggested that iron-bound *H. pylori* Fur recognizes a poorly defined conserved A/T-rich consensus Fur box sequence (AATAATNNTNA) (Merrell *et al.* 2003), which is quite different from the *E. coli* Fur box (GATAATGAT[A/T]ATCATTATC) (de Lorenzo *et al.* 1987). Interestingly, however, Bereswill, et al. observed that *H. pylori* Fur is able to complement an *E. coli fur* mutant strain (Bereswill *et al.* 1999), and herein we found that *E. coli* Fur provided the most efficient heterologous complementation in the *H. pylori Δfur* strain (Fig. 2). Studies directed at understanding the Fur box recognized by iron-bound *H. pylori* Fur may reveal greater conservation than previously appreciated. Additionally, given that the current binding sequence for *apo*-Fur (TTNNNNNNNANNTNNNNNAATNNTNNNANNN) (Delany *et al.* 2001) is even less well defined, there is clearly much to learn about how *H. pylori* Fur identifies its target genes.

Interestingly, despite the relatively high degree of conservation among bacterial Fur species, this conservation does not necessarily translate into the individual Fur species showing compatible binding and functional capabilities. Indeed this may be due to subtle but important structural differences among the various protein species. For instance, even though *V. cholerae* and *P. aeruginosa* share 51.3% identity and 70.7% similarity, recent crystal structures of each protein revealed that their DNA binding regions show very different orientations (Pohl *et al.* 2003; Sheikh and Taylor 2009), which likely greatly affects Fur function and DNA recognition. Additionally, regions that are implicated for being necessary for metal binding in one species (*V. cholerae*) appear to be nonessential in a closely related species with 96% identity (*Vibrio harveyi*) (Sun *et al.* 2008). Therefore, while Fur may be found in many Gram positive and Gram negative bacterial species and regulate many similar types of genes, conservation of motifs and domains does not guarantee conservation of function.

Given its capacity for chronic infection, *H. pylori* has clearly evolved to exist in the dynamic gastric niche. However, interestingly the bacterium encodes few two component systems (Wang *et al.* 2006), a paucity of general transcriptional regulators, and, to date, only four identified sRNAs (Xiao *et al.* 2009; Xiao *et al.* 2009; Xiao *et al.* 2009). Given this regulatory deficit, to successfully respond to the environmental stressors found in the stomach, the transcriptional regulators encoded by *H. pylori* may have evolved to assume more complex mechanisms of regulation to compensate for their limited numbers. For example, while *apo*-Fur regulation has not been identified in other species, certain genes in *E. coli*, *P. aeruginosa*, and *V. cholerae* are known to be repressed in a Fur-dependent manner when iron is depleted (Litwin and Calderwood 1994; Wilderman *et al.* 2004; Masse *et al.* 2007). However, in these organisms, this regulation is mediated by the Fur-regulated sRNA RyhB (Masse and Gottesman 2002). Similar to *apo*-Fur regulation of *sodB* and *pfr* in *H. pylori* (Ernst *et al.* 2005), RyhB has been shown to regulate *sodB* and ferritin expression in *E. coli* (Dubrac and Touati 2000; Masse and Gottesman 2002; Masse *et al.* 2007), *P.*

aeruginosa (Wilderman *et al.* 2004), and *V. cholerae* (Mey *et al.* 2005). However, to date, no RhyB homolog has been identified in *H. pylori* (Delany *et al.* 2001). Thus, perhaps in an effort to compensate for the lack of *ryhB*, in *H. pylori* Fur may have evolved to acquire dual iron-bound and *apo*-Fur regulatory functions. Conversely, one could predict that those organisms with RhyB would not need to acquire *apo*-Fur regulation. Thus, the unique ability of *H. pylori* Fur to function as an *apo*-regulator in the absence of its iron cofactor may be a sign of this evolution. The data presented here support this idea since none of the heterologous Fur proteins were able to complement *apo*-Fur regulation despite a moderate degree of identity and similarity. While the regions of *H. pylori* Fur that impart the unique ability for *apo*-regulation are not immediately evident, Carpenter and Merrell recently showed that mutations in E90 and H134, which lie in residues predicted to be *H. pylori* Fur metal binding sites, result in an altered *apo*-Fur phenotype (Carpenter and Merrell, unpublished data). These residues are completely conserved within *C. jejuni*, *D. vulgaris*, *E. coli*, *P. aeruginosa*, and *V. cholerae* (Figure 1), suggesting that the presence of these sites alone does not confer *apo*-Fur regulation. In all, these data highlight how much remains to be understood about *apo*-Fur regulation and the need for continued study of this unique regulatory mechanism in this medically important pathogen.

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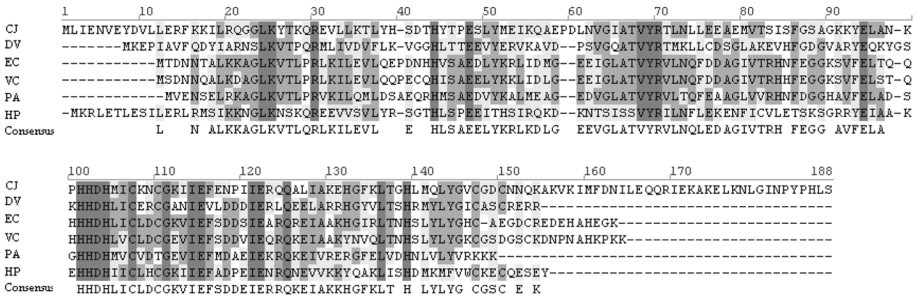

Figure 1.

Alignments of Fur coding sequences. Amino acid sequence alignment of Fur from *C. jejuni* (CJ), *E. coli* (EC), *D. vulgaris* (DV), *H. pylori* (HP), *P. aeruginosa* (PA), and *V. cholerae* (VC). Identical residues are indicated by dark grey, conservative residues by medium grey, and similar residues by light grey. The alignment was constructed using AlignX software (Vector NTI, Invitrogen, USA).

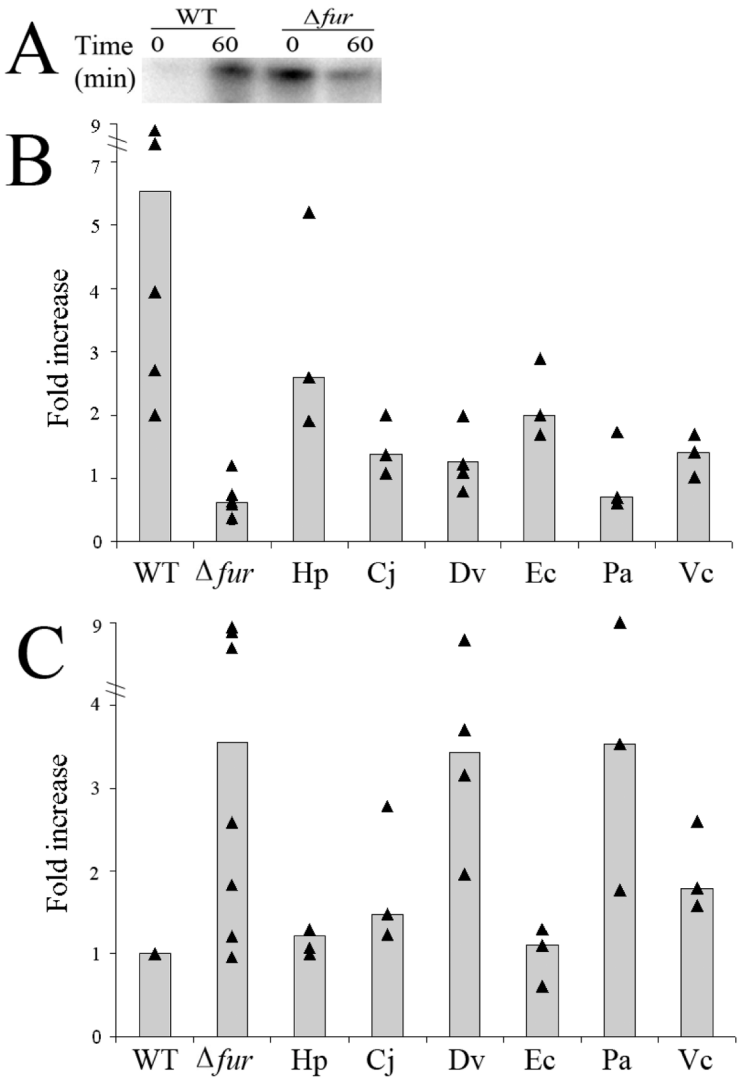

Figure 2.

Determination of the ability of the heterologous constructs to complement iron-bound Fur regulation of *amiE*. Wild type *H. pylori* (WT), *Δfur H. pylori* (*Δfur*), and *Δfur H. pylori* carrying the heterologous Fur constructs from *C. jejuni* (Cj), *D. vulgaris* (Dv), *E. coli* (Ec), *H. pylori* (Hp), *P. aeruginosa* (Pa), and *V. cholerae* (Vc) were grown to exponential phase in iron replete liquid media. On the subsequent day, one half was used for RNA isolation (t_0). The other half was exposed to iron deplete conditions for one hour by the addition of 200 μM dpp prior to isolation of the RNA (t_{60}). Each triangle represents a biologically independent set of RNA. Median fold change is represented as a bar for each strain. (A) RPA using an *amiE* riboprobe showed classical Fur dependent changes in *amiE* expression. (B) Graphic depiction of the fold increase in expression of *amiE* calculated by comparing the relative amount of protected riboprobe in the iron deplete (t_{60}) condition to the iron replete condition (t_0). (C) Basal level of repression of *amiE* at t_0 in each of the heterologous strains as compared to WT.

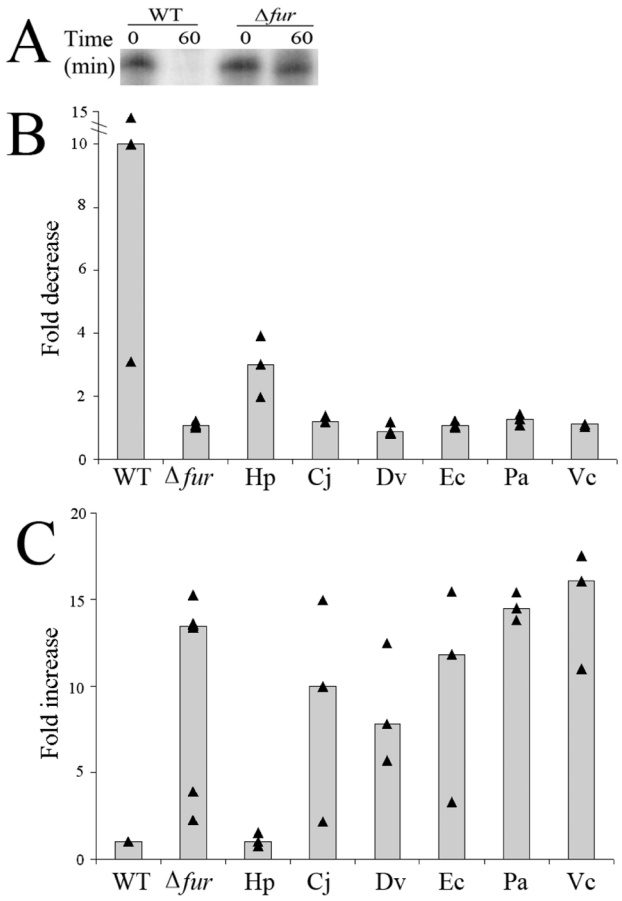

Figure 3.

Determination of the ability of the heterologous constructs to complement *apo*-Fur regulation of *pfr*. Wild type *H. pylori* (WT), *Δfur H. pylori* (*Δfur*), and *Δfur H. pylori* carrying the heterologous Fur constructs from *C. jejuni* (Cj), *D. vulgaris* (Dv), *E. coli* (Ec), *H. pylori* (Hp), *P. aeruginosa* (Pa), and *V. cholerae* (Vc) were grown to exponential phase in iron replete liquid media. On the subsequent day, one half was used for RNA isolation (t_0). The other half was exposed to iron deplete conditions for one hour by the addition of 200 μM dpp prior to isolation of the RNA (t_{60}). Each triangle represents a biologically independent set of RNA. Median fold change is represented as a bar for each strain. (A) RPA using a *pfr* riboprobe showed *apo*-Fur dependent changes in *pfr* expression. (B) Graphic depiction of the fold decrease in expression of *pfr* calculated by comparing the relative amount of protected riboprobe in the iron replete (t_0) condition to the iron deplete condition (t_{60}). (C) Basal level of repression of *pfr* at t_{60} in each of the heterologous strains as compared to WT.

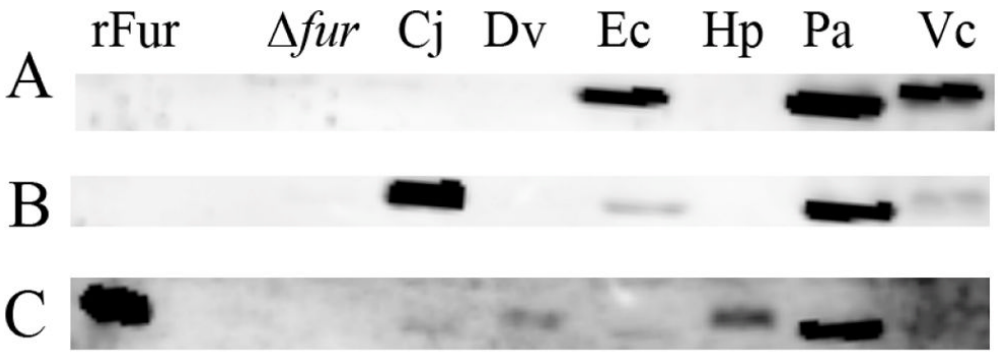

Figure 4.

Anti-Fur Western blot. Purified recombinant *H. pylori* G27 Fur (rFur), and equal concentrations of lysates from *Δfur H. pylori* (*Δfur*) and *Δfur H. pylori* carrying the Fur constructs from *C. jejuni* (Cj), *D. vulgaris* (Dv), *E. coli* (Ec), *H. pylori* (Hp), *P. aeruginosa* (Pa), and *V. cholerae* (Vc) were subjected to Western blot analysis. (A) Martha 2472 rabbit polyclonal anti-*P. aeruginosa* Fur antibody was used to detect *E. coli*, *P. aeruginosa*, and *V. cholerae* Fur. (B) Polyclonal rabbit anti-*C. jejuni* Fur antibody was used to detect *C. jejuni*, *E. coli*, *P. aeruginosa*, and *V. cholerae* Fur proteins. (C) Polyclonal rabbit anti-*H. pylori* Fur antibody was used to detect recombinant *H. pylori* Fur, *C. jejuni*, *D. vulgaris*, *E. coli*, *H. pylori* and P. aeruginosa Fur. These data are representative of multiple independent experiments.

TABLE 1

Plasmids and strains used in this study

Plasmid or strain	Description	Reference
Plasmids		
pDSM226	pGEM T-easy:: *H. pylori fur*	(Carpenter *et al.* 2007)
pDSM340	pTM117:: *H. pylori fur*	(Carpenter *et al.* 2007)
pDSM515	pTM117:: Hp *V. cholerae fur*	This study
pDSM521	pGEM T-easy:: Hp *E. coli fur*	This study
pDSM522	pGEM T-easy:: Hp *C. jejuni fur*	This study
pDSM523	pGEM T-easy:: Hp *V. cholerae fur*	This study
pDSM526	pTM117:: Hp *E. coli fur*	This study
pDSM560	pTM117:: Hp *C. jejuni fur*	This study
pDSM642	pGEM T-easy:: Hp *P. aeruginosa fur*	This study
pDSM652	pTM117:: Hp *P. aeruginosa fur*	This study
pDSM755	pGEM T-easy:: Hp *D. vulgaris* Hildenborough *fur*	This study
pDSM758	pTM117:: Hp *D. vulgaris* Hildenborough *fur*	This study
H. pylori strains		
DSM300	G27 *Δfur*:: cat, Cm^r	(Carpenter *et al.* 2007)
DSM343	G27 *Δfur* (pDSM340), Kan^r Cm^r	(Carpenter *et al.* 2007)
DSM554	G27 *Δfur* (pDSM526), Kan^r Cm^r	This study
DSM557	G27 *Δfur* (pDSM523), Kan^r Cm^r	This study
DSM583	G27 *Δfur* (pDSM560), Kan^r Cm^r	This study
DSM712	G27 *Δfur* (pDSM652), Kan^r Cm^r	This study
DSM761	G27 *Δfur* (pDSM758), Kan^r Cm^r	This study

TABLE 2

Primers used in this study

Primer[b]	Sequence (5′-3′)[a]	Reference
RPA primers		
amiE-RPA-F	GGTTTGCCTGGGTTGGAT	(Gancz *et al.* 2006)
amiE-RPA-R	GATTTTGCGGTATTTTG	(Gancz *et al.* 2006)
pfr-RPA-F	GCGGCTGAAGAATACGAG	(Carpenter *et al.* 2007)
pfr-RPA-R	CTGATCAGCCAAATACAA	(Carpenter *et al.* 2007)
Hp fur RPA F	GAGCGCTTGAGGATGTCTATC	(Carpenter *et al.* 2007)
Hp fur RPA R	GTGATCATGGTGTTCTTTAGC	(Carpenter *et al.* 2007)
Cj fur RPA F	CCTGATTTAAATGTAGGAATTGC	This study
Cj fur RPA R	AAAGCTGCATCAAATGCCCTG	This study
Dv fur RPA F	CAACAGCCTCAAGGTGAC	This study
Dv fur RPA R	GTTCGATGTCGTCGTCGA	This study
Ec fur RPA F	GGAGCCGGACAACCATC	This study
Ec fur RPA R	CGCTTCGATGGAATCATC	This study
Pa fur RPA F	GACTCGGCCGAGCAAC	This study
Pa fur RPA R	ATTTCCTTCTGGCGCTTCTC	This study
Vc fur RPA F	CTCCCACGGCTTAAGATTTTAG	This study
Vc fur RPA R	GACGTTGTTCAATCACATCG	This study
SOE primers		
FurCF1 (XbaI)	TCTAGAAAGGCTCACTCTACCCTATT	(Carpenter *et al.* 2007)
Cj furR (SalI)	GTCGACAAATGAGGATAAGGATTGATCCC	This study
Cj SOE F	CATTTTACGGATAAGGGAAATATCAGCATGCTGATAGAAAATGTGGAATATGATG	This study
Cj SOE R	CATCATATTCCACATTTTCTATCAGCATGCTGATATTTCCCTTATCCGTAAAATG	This study
Dv fur R	GGTACCTCGTTCACCCGCAC	This study
Dv SOE F	CATTTTACGGATAAGGGAAATATCAGCATGAAGGAACCCATCGCCGTATTTC	This study
Dv SOE R	GAAATACGGCGATGGGTTCCTTCATGCTGATATTTCCCTTATCCGTAAAATG	This study
Dv SOE F2	GAAGCTCCTGTGCGACTCAGGTCTCGCCAAGGAAGTGC	This study
Dv SOE R2	GCACTTCCTTGGCGAGACCTGAGTCGCACAGGAGCTTC	This study
Ec FurR (SalI)	GTCGACGATAAGGTCTGGCAGGAAATTCGC	This study
Ec SOE F	CATTTTACGGATAAGGGAAATATCAGCATGACTGATAACAATACCGCCCTAAAGAAAG	This study

Primer[b]	Sequence (5′-3′)[a]	Reference
Ec SOE R	CTTTCTTTAGGGCGGTATTGTTATCAGTCATGCTGATATTTCCCTTATCCGTAAAATG	This study
Pa furR (KpnI)	GGTACCTGGCCGCCCAGAACTGAAC	This study
Pa SOE F	CATTTTACGGATAAGGGAAATATCAGCATGGTTGAAAATAGCGAACTTCGAAAAGC	This study
Pa SOE R	GCTTTTCGAAGTTCGCTATTTTCAACCATGCTGATATTTCCCTTATCCGTAAAATG	This study
Vc FurR (SalI)	GTCGACAACCCACCATTCGGTGGG	This study
Vc SOE F	CATTTTACGGATAAGGGAAATATCAGCATGTCAGACAATAACCAAGCGCTAAAGG	This study
Vc SOE R	CCTTTAGCGCTTGGTTATTGTCTGACATGCTGATATTTCCCTTATCCGTAAAATG	This study

[a] Restriction endonuclease sites are underlined

[b] Important restriction sites are included in parentheses

Table 3

Percent identity and similarity of bacterial Fur amino acid sequences as compared to *H. pylori* Fur[a]

	Identity to *H. pylori*	**Similarity to *H. pylori***
C. jejuni	32.6%	52.2%
D. vulgaris	30.5%	49.3%
E. coli	29.1%	52.7%
P. aeruginosa	26.5%	54.0%
V. cholerae	25.6%	52.0%

[a]Identity and similarity were calculated using MatGat 2.0 (Campanella *et al.* 2003).

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