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Apo and Iron Bound Fur Repression and the Role of Fur *in vivo*

by

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Abstract

Title of Dissertation:

Apo and Iron Bound Fur Repression and the Role of Fur *in vivo*

Shana Melody Miles, Doctor of Philosophy, 2010

Thesis Directed by:

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Helicobacter pylori is a Gram negative neutrophile that persistently colonizes the dynamic gastric environment and is associated with the development of a spectrum of gastric pathology ranging from gastritis to invasive adenocarcinoma. Despite its fluctuating gastric niche, *H. pylori* genomic studies have revealed surprisingly few regulatory factors and two component systems. Of these identified factors, the Ferric Uptake Regulator (Fur) has been shown to control a diverse regulon. Even though Fur has been identified and characterized in both Gram positive and negative bacteria, to date, *H. pylori* Fur appears unique in its ability to regulate genes in its *apo* form. The first section of this study examined the ability of other bacterial Fur to complement apo-Fur regulation in *H. pylori*. Results from this study showed that *Campylobacter jejuni, Desulfovibrio vulgaris, Escherichia coli, Pseudomonas aeruginosa,* and *Vibrio cholerae* Fur are unable to complement apo-Fur regulation within the context of *H. pylori*. Next

we examined the role of Fur in a gerbil model of infection. The results indicate that Fur is important for establishing colonization and plays a role in the development and progression of disease. The data presented here provide the basis for future studies to examine the role of iron-bound and *apo*-Fur *in vivo.* In *H. pylori*, while Fur is nonessential, it is crucial in the initial colonization and temporal progression and severity of disease.

Dedications

To my sister, Katherine,

for all of your love and helping me keep perspective.

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Chapter One

Introduction

Helicobacter pylori

Epidemiology

Helicobacter pylori was first cultured by Barry Marshall and Robin Warren in 1982 (145) and has since become the subject of tremendous study. This gastric pathogen is a microaerophilic Gram-negative bacterium that colonizes over half of the world's population (23). In terms of distribution, *H. pylori* infection occurs worldwide, but the prevalence differs significantly between countries and within their economic stratifications (136, 154, 181). The prevalence of *H. pylori* infection is lower in North America, and Western and Northern Europe as compared to Asia and Eastern Europe (1). Malaty *et al* found that there was a significant difference in infection in monozygotic twins reared apart with increased rates of *H. pylori* infection in those raised in lower socioeconomic status (138). Moreover, there was no effect of adult socioeconomic status on infection rates which further supports the hypothesis of childhood acquisition (138).

Currently only humans and non human primates have been definitively shown to serve as reservoirs (76, 97), as such, the source of *H. pylori* infection remains a source of controversy. Insects (93, 94, 242), animals (93, 94, 242), and water (124) have been proposed as potential reservoirs for *H. pylori*. However, the validity of those studies and the possibility of these sources serving as reservoirs have been questioned. While *H. pylori* DNA is able to be detected in various water sources (109, 110, 200), to date there have been no successful attempts to culture *H. pylori* from water samples. In short, while

there has been extensive work to identify an environmental reservoir of *H. pylori*, none has been definitively implicated.

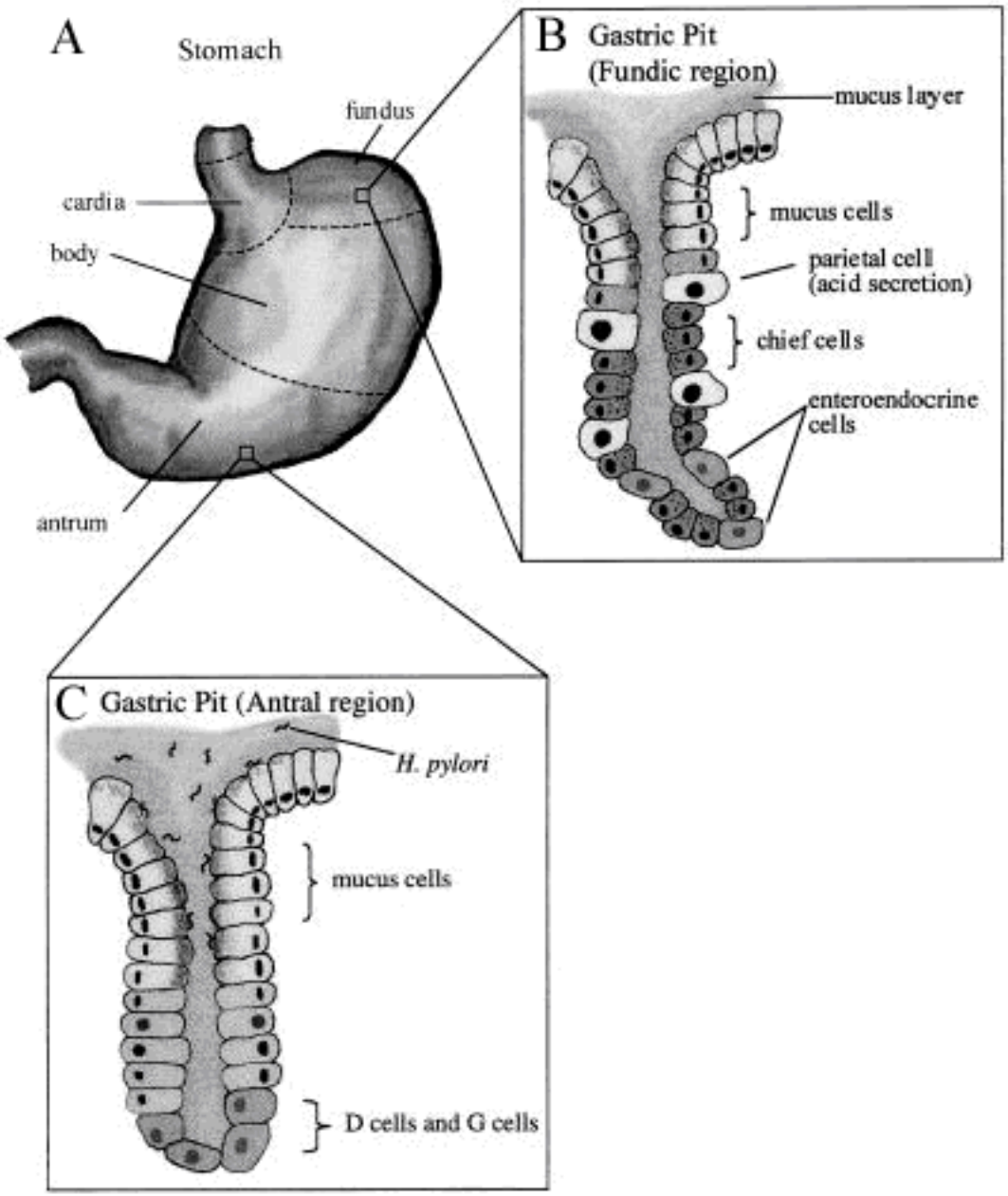
The mode of transmission of *H. pylori* among infected individuals is also an area of controversy. *H. pylori* has been detected in the oral cavity (70, 140) and in feces (141, 164) suggesting either an oral-oral or fecal-oral route. However, attempts to culture *H. pylori* from either of these sites have been largely unsuccessful (156, 165). Even so, *H. pylori* can be isolated from the oral cavity of infected individuals after induction of vomiting (178). Thus, even though the oral cavity may not be a true site of *H. pylori* colonization, its transient culturable presence in the mouth raises the possibility that oral-oral transmission may play a role in the documented increased risk of transmission within families (15, 81, 191, 234), between spouses (27), and in institutionalized subjects (19, 123, 126). Additionally, cultural practices may play a role in the differences in oral transmission and may partially account for the differences in global distribution of *H. pylori* infection and disease. For example, Asian chopstick use and communal eating and the premastication of food by African mothers to feed their children have been associated with increased risk of *H. pylori* transmission (5, 38). Conversely, if oral-oral transmission was the key route of *H. pylori* transmission, a corresponding increase in the prevalence of *H. pylori* in dentists and dental workers might be expected. Epidemiological evidence, however, has not supported such an increase (132, 137). Taken together, there is clearly much to learn about *H. pylori* transmission.

Gastric niche

The stomach has been recognized as an acidic digestive chamber since 1648 (190) and is generally divided into four sections: cardia, fundus, corpus, and antrum

(Figure 1). These sections are then further divided into four layers: the mucosa, submucosa, muscularis externa, and serosa (221). The mucosa forms glandular pits with distinct functional and histological features between the corpus and antrum. In the fundus and corpus, the mucosa is composed of the acid secreting parietal cells, pepsinogen secreting chief cells, and mucus neck cells intercalated with enterochromaffin-like cells that produce a precursor of histamine (221). In contrast to the fundus and corpus, the non-acid producing antrum is responsible for the secretion of mucus, bicarbonate, gastrin, and somatostatin (221).

Figure 1. Depiction of the human stomach.



(235)

A. The four sections of the stomach.

B. Graphic representation of the cellular structures in the mucosa of the fundus and corpus.

C. Graphic representation of the mucosal area of the antrum infected with *H. pylori.*

In the stomach, pH is influenced by both acid secretion and content. In the acid producing corpus of the human stomach, the median daytime pH ranges from 1.8-4.5 while the pH ranges from 1.6-2.6 in the antrum (150). While patterns of gastric acidity vary between individuals, a circadian rhythm of acidity occurs with the highest acid output in the evening and lowest in the morning (165). This acidic pH also aids in the absorption and availability of many nutrients (221).

Interestingly, food has a buffering effect on gastric acid and raises the pH of the stomach. However, different areas of the stomach respond quite differently. After ingestion, the corpus of the stomach was shown to increase to pH 4.5 and then return to its basal level pH 3.5 hours later whereas the antrum showed a much smaller magnitude of change (150). This suggests that the pH is relatively low and stable in the antrum (150). However, the delicate balance of the pH of the corpus and antrum can go awry. For example, chronic gastritis may cause the replacement of the acid producing mucosa of the corpus with increased gastrin secreting antral mucosal cells which then predisposes the corpus-antrum junction to ulcer formation (72).

Diagnosis, treatment, and disease associated with H. pylori *infection*

H. pylori can be detected in patients noninvasively through the urea breath test, blood antibody test, or stool antigen test. However, the most reliable method of detection remains biopsy followed by a rapid urease test and culture (135, 222). Once detected, *H. pylori* is treated with a 10 day therapy consisting of a proton pump inhibitor, clarithromycin, and amoxicillin (160). Alternatively quadruple therapy (triple therapy with the addition of bismuth) is sometimes indicated in the face of increasing antibiotic resistance (89, 90).

There is a broad range of disease severity associated with *H. pylori* infection; these pathologies range from subclinical to the development of gastric cancer (159). Following the initial infection and colonization of *H. pylori* in the gastric mucosa, the underlying epithelium begins to show signs of infection. This typically includes the classic hallmarks of gastritis and includes mucin depletion, accumulation of PMNs in the epithelium, and edema of the lamina propria (143, 218). The initial acute phase is associated with neutrophilic infiltration, hypochlorhydria, and lack of ascorbic acid secretion (219). This phase is usually subclinical and short lived and, if the infection is not cleared, progresses to chronic gastritis (218). Chronic gastritis is denoted by the presence of lymphocytes, plasma cells, and characteristic lymphoid follicles in the gastric mucosa (259).

Variation in the severity of gastritis among patients is in part due to differences in the cytotoxin-associated gene A, *cagA* (43, 240). CagA is *H. pylori* encoded and injected into the host cell through a type IV secretion system encoded by the *cag* pathogenicity island (11, 175, 205). Detailed molecular analysis has shown that most of the *cag* genes are necessary for the induction of the proinflammatory cytokine, IL-8, that results in the infiltration of neutrophils to the infection site (46, 47). Additionally, there is a potent IL-8 response mediated by IL-1 that is released by macrophages in response to bacterial LPS and from PMNs (122). While *H. pylori* infection is cleared in a minority of patients, the immune response of most individuals fails to clear the infection and these individuals develop an active chronic gastritis (218). This chronic induction of IL-8 and the resulting chronic inflammation is believed to exacerbate the development of severe disease. (48).

H. pylori associated gastritis is typically more pronounced in the antrum than in the corpus of the stomach (148). This predominance is thought to be due to the localized lower pH around the parietal cells in the acid producing corpus and is supported by several studies (152, 172, 227). For example, when duodenal ulcer patients undergo vagotomy, thus reducing the output of acid, an increase in inflammation of the stomach corpus occurs (171). Furthermore, when patients with *H. pylori* infection are treated with proton-pump inhibitors to suppress acid secretion, colonization of the stomach corpus predominates (227).

Location of the *H. pylori* induced gastritis confers differential increased risk of developing ulcers and gastric cancer. The development of duodenal ulcers is associated with antrum predominant gastritis (212, 213), whereas there is an increased risk of gastric ulcers and gastric cancer in individuals displaying predominant stomach corpus colonization and gastritis (211). Duodenal and gastric ulcers are both correlated with *H. pylori* infection; nearly 100% of patients with duodenal ulcers and 80-95% of patients with gastric ulcers are infected with *H. pylori* (115, 116). The association of *H. pylori* with ulcer development is further strengthened by the fact there was no relapse of ulcers in patients receiving antibiotic treatment for eradication of *H. pylori* (40).

Mucosa-associated lymphoid tissue (MALT) B-cell lymphomas were first described during the same year as *H. pylori* (112, 145). While the stomach typically lacks lymphoid tissue, the presence of lymphoid cells is associated with colonization by *H. pylori* (125). Indeed, the chronic inflammatory response to *H. pylori* infection is characterized by the presence of lymphoid follicles and an inability to clear the infection (86). These follicles subsequently provide the platform for development of mucosa-

associated lymphoid tissue (MALT) in which gastric marginal zone lymphoma can occur (192). After antibiotic treatment to eliminate *H. pylori*, up to 80% of patients with MALT lymphoma see full remission (125). Consequently, *H. pylori* treatment is the first line treatment for low grade gastric MALT lymphomas (139). However, even though there is a strong association between chronic *H. pylori* infection and gastric MALT lymphoma (113), the vast majority of those infected with *H. pylori* never develop this disease (177, 254).

Intestinal metaplasia has also been correlated with *H. pylori* infection (44, 45) and is characterized by the replacement of normal gastric mucosa with intestinal mucosa, which is considered preneoplastic (29). The progression from gastritis to the development of intestinal metaplasia is still controversial and, unlike MALT lymphoma, there is not a consensus on whether eradication of *H. pylori* results in decreased progression or reversal of intestinal metaplasia (131, 166, 241, 243).

Based on epidemiological data alone, in 1994 the World Health Organization classified *H. pylori* as a group I (definite) carcinogen (2). Gastric cancer is the second most common cause of global cancer deaths (49) and areas with the highest prevalence of *H. pylori* infection have a corresponding high rate of gastric cancer (3, 49). Gastric cancer is associated with chronic inflammation (88) and increased rates of gastric epithelial proliferation (179). During inflammation, the activated inflammatory cells lead to the production of reactive oxygen species (ROS) which in turn further upregulate the production of IL-8 (207). It is believed that the dual effect of ROS inflicted DNA damage as well as the inflammation induced increased cell turnover leads to the development of gastric cancer (68, 92). This is further supported by the fact that *H.*

pylori strains carrying the *cag* genes, which promote IL-8 induction, are twice as likely to induce cancer as their *cag* negative counterparts (24).

Adaptation

The success of *H. pylori* despite its harsh environment and large fluctuations in nutrient availability, suggests that *H. pylori* must be able to regulate gene expression to survive in its niche. Despite this necessity to adapt, analysis of the genome sequences of the four sequenced *H. pylori* strains reveals the presence of only a few classical transcriptional regulators and two-component systems (7, 14, 201, 237). Clearly, *H. pylori* has evolved to survive in its dynamic gastric niche and to survive in this harsh environment, *H. pylori* must be able to acclimate to its acidic environment (142, 182, 195). This fact is remarkable given the extremes in pH and the fact that *H. pylori* is considered a neutrophile (193). Even in an acidic environment (as low as pH 2.5) *H. pylori* is able to maintain a periplasmic pH of 6.1 (142).

While bacteria like *E. coli, S. typhimurium, V. cholerae,* and *Y. enterocolitica* are able to survive the transit through the human gastric environment, only *H. pylori* is able to effectively colonize this niche. One of the tools that *H. pylori* utilizes to respond to the acidic environment is urease, which is an enzyme that elevates pH by catalyzing hydrolysis of urea to produce the basic molecules ammonia and carbon dioxide. Urease has been shown to be crucial for low pH survival *in vitro* and in animal models (60, 61, 189). Urease is encoded as part of a gene cluster. Within the urease gene cluster is *ureI*, which encodes for an acid-activated urea channel that is responsible for internalization of urea into the bacterial cytoplasm and is necessary for maintenance of near neutral

intrabacterial pH (9, 189, 216). Urease is not unique to *H. pylori* and is sometimes considered a virulence factor in other bacteria as well (85, 117). However, unlike most other bacterial ureases, *H. pylori* urease can be found not only in the cytoplasm but also adherent to the cell surface and shed into the environment (184). The theme of the importance of ammonia production for survival in low pH is not unique to urease as *H. pylori* also uses amidase and foramidase to generate ammonia to buffer its environment (194, 214, 215, 223, 224).

Within the stomach, *H. pylori* colonizes the gastric mucosa. Here, the mucus layer is an important barrier that protects the stomach lining from acidity and from pepsin proteolytic digestion (6). The pH across the mucus layer varies from a pH of 2 at the gastric lumen to a more neutral pH of 5 at the epithelial surface (6). Additionally, within the mucus layer, there are chemical gradients (203, 233), a bicarbonate gradient (75, 187, 188), and, when infected with *H. pylori*, a urea/ammonium gradient (121). Here, the gastric mucin, which is responsible for the viscoelastic response of the gastric mucus, undergoes a reversible pH dependent transition in viscosity which results in an increased viscosity at low pH (33, 36). These gradient changes and consequent effects on the mucus viscosity affect *H. pylori* chemotaxis (202), motility (37), and orientation (10) of the bacterium.

H. pylori appears to affect the thickness of the mucosal barrier of the stomach (4). In a study on human biopsies, although age and sex had no influence on the mucus thickness, there was a correlation between high colonization density of the less pathogenic *cagA* negative strains and thinning of the mucus layer (4). The increased thickness in those infected with a high density of *cagA* positive strains could potentially

be due to increased inflammation. The mechanisms *H. pylori* has developed to not only survive but colonize this callous environment are remarkable.

Iron

In most organisms, and particularly in bacterial pathogens that must compete with their host for this nutrient, iron is essential (186). Iron is extremely important because it plays a role in respiration, electron transport, and is a required cofactor for many enzymes (198). However, too much free iron also leads to the Fenton reaction, which results in DNA-damaging, protein denaturing hydroxyl radicals (95). Dealing with the need to establish iron homeostasis is of increased difficulty in *H. pylori* because of its site of colonization. The typical iron concentration of gastric juice is 0.3 mg/100mL but can vary widely based on food consumption (20). Additionally, ferrous iron (Fe^{+2}) is more readily available at pH 5 (10^{-3} M) than at pH 7 ($1.4x10^{-9}$ M) (8). This increased availability of ferrous iron forces *H. pylori* to adapt to the increased threat of iron overload during changes in stomach pH.

The oxidative state of iron is crucial in the ability of iron to be used as a cofactor, its readiness for transport and absorption, and its use in enzymatic reactions (260). Most dietary iron is found in its ferric (Fe^{+3}) form and must be reduced to ferrous (Fe^{+2}) iron for absorption in the intestine (260). Since ferric iron is usually complexed with heme, transferrin, lactoferrin, or ferritin within the host (8, 245), it must be actively transported across the bacterial outer and cytoplasmic membranes. As such, bacteria have developed iron uptake systems to aid in iron acquisition. *H. pylori* has homologues of the FecA, FrpB, and TonB/ExbB/ExbdD proteins, which are important for iron uptake (245, 248).

Interestingly, there appears to be redundancy in the iron acquisition mechanisms of *H. pylori* since mutations in *fecA* (allows recognition of ferric iron) or *tonB* (generates energy for transport across the outer membrane) do not affect colonization (248).

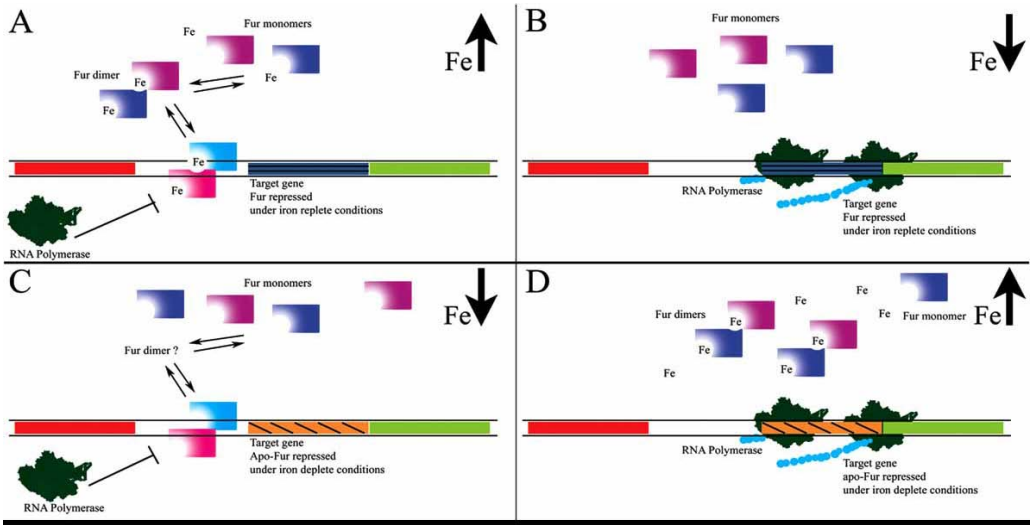
The need to balance iron acquisition and storage also influences host disease. In bacteria, genes that encode factors for acquiring and storing iron are often considered virulence factors (26). Interestingly, iron overload has been correlated with an increased susceptibility to bacterial infections (105, 119, 231) showing that the delicate balance of iron acquisition is of importance for bacterial colonization. An example of the importance of iron in host disease is iron deficiency anemia. Iron deficiency anemia is associated with both symptomatic and asymptomatic *H. pylori* infection (16, 169). This anemia is not responsive to iron replacement therapy and has been shown to be resolved after eradication of *H. pylori* (16, 34). Interestingly, there does not appear to be any additional benefit from coadministration of *H. pylori* eradication therapy with iron therapy (87).

While the exact mechanism of the development of *H. pylori* associated iron deficiency anemia is unknown, it has been shown that strains from anemic patients display increased uptake of iron (258) and contain polymorphisms in the ferrous iron transporter, *feoB* (114) suggesting that host-pathogen competition is a factor in disease development. However, development of *H. pylori* induced iron deficiency anemia could be more subtle. The interplay of decreased gastric and mucosal ascorbic acid secretions likely result in decreased reduction of ferric iron to the more readily absorbed ferrous iron. Additionally, development of severe gastritis likely leads to host cell damage and loss of cellular iron stores (39, 73).

Ferric Uptake Regulator (Fur)

The precarious balance between acquiring enough but not too much iron in *H. pylori*, as well as in many other bacterial pathogens, is mediated by the Ferric Uptake Regulator (Fur). Regulation of intracellular iron homeostasis by Fur has been investigated in several Gram-negative and Gram-positive bacteria. Indeed, Fur is found in a diverse number of bacterial species and has been extensively studied since it was first identified in *Salmonella typhimurium* in 1978 (67). In that original study, a *S. typhimurium fur* mutant was shown to express high levels of iron-enterochelin and iron uptake systems (67). Shortly thereafter, *E. coli fur* mutants were shown to display constitutive expression of three iron uptake systems, which are usually upregulated only in iron depleted conditions (99). This phenomenon of Fur regulating iron uptake systems has since been documented in a wide range of Gram-positive and Gram-negative bacteria (67, 74, 98, 107, 174, 247).

Figure 2. Schematic of Fur regulation



(252)

Iron-bound Fur regulation:

A. When iron is replete, iron-bound Fur represses gene expression.

B. When iron is deplete, gene transcription proceeds.

apo-Fur regulation:

C. When iron is deplete, gene transcription is repressed.

D. When iron is replete, gene transcription proceeds.

Mechanistically, in the classical form of Fur regulation, Fur acts as a repressor only when bound to iron (Figure 2A, 2B). Structural studies have shown that the *E. coli* Fur protein appears mainly as a dimer in solution (173) and the biologically active form of Fur is suggested to be at least a dimer (26). In both *E. coli* and *H. pylori* Fur, the C-terminal domain is necessary for multimerization of the protein (53, 226) and, in *H. pylori*, studies have shown that iron-responsiveness of Fur can be eliminated through mutation of two residues predicted to be C-terminal iron-binding sites (53).

Once dimerized, iron-bound Fur binds to promoter elements called Fur boxes (69). The bound Fur prevents the binding of RNA polymerase, thus, repressing expression of the gene. While Fur boxes vary between organisms, they tend to be A/T rich. In *E. coli* there is a 19 bp well defined Fur box (GATAATGATAATCATATC). *B. subtilis* has a 15 bp inverted repeat which partially resembles the 19 bp *E. coli* Fur box (12, 13, 51). In *Y. pestis*, the Fur box consists of two inverted repeats of AATGATAAT separated by a single base (84). The iron-bound Fur boxes of *H. pylori* tend to overlap the -10 and -35 sites and, in contrast to these other organisms, are not well conserved (155). This suggests that the recognition of target sites by *H. pylori* Fur occurs in a different manner than that of other organisms.

H. pylori Fur appears unique in that it also utilizes *apo*-Fur regulation (18, 66). In this form of regulation, Fur acts as a repressor even in the absence of its iron cofactor (Figure 2C, 2D). While microarray analyses of *C. jejuni* (106) and *D. vulgaris* (17) suggest that they may also be capable of *apo*-Fur regulation, to date, *apo*-Fur regulation has only been definitively shown to occur in *H. pylori*. Currently, there are 16 genes that are predicted to be regulated by *apo*-Fur in *H. pylori* (65). Two of these genes, the

superoxide dismutase, *sodB* (66), and the iron storage gene, *pfr* (18, 35, 55), have been definitively shown to be directly regulated by *apo*-Fur. Footprinting analysis of the *pfr* promoter showed the presence of three Fur boxes of varying affinities (55) while analysis of the *sodB* promoter showed a single Fur box (66). Similar to the iron-bound Fur boxes, the Fur binding sites of these target genes overlap the -10 and -35 promoter elements and block the RNA polymerase (55, 65). Additionally, there appears to be very little homology between the *apo*-Fur boxes (55, 65).

The distinctive *apo*-Fur regulation of *H. pylori* has not been accepted without debate. Previously, there were several instances of genes that appeared to be regulated by *apo*-Fur in *E. coli*, *P. aeruginosa*, and *V. cholerae* (133, 147, 253). However, it is now known that regulation of these genes is mediated by the small RNA (sRNA) RyhB (146), which is often Fur regulated. sRNAs can alter the stability of mRNA or its ability to be translated through base pairing with target transcripts. Thus, sRNAs have been suggested as an alternative mechanism to regulate the genes in the *apo*-Fur regulon (228). RyhB is expressed when iron is deplete and, with the assistance of the RNA binding protein Hfq, binds to its mRNA target and prevents translation (146, 163). Interestingly, both *sodB* and *pfr* which have been shown to be regulated by *apo*-Fur in *H. pylori* (55, 66), are regulated by RyhB in *E. coli* (59, 146, 147), *P. aeruginosa* (253), and *V. cholerae* (156). However, no RhyB homologue has been identified in *H. pylori*. Until recently, only four sRNAs had been identified in *H. pylori*: NAT-39, NAT-67, IG-443, and IG-524 (256, 257). NAT-39 and NAT-67 are complementary to genes encoding a protein involved in iron uptake and transport (*frpB, ceuE*) (257). IG-443 is encoded in the intergenic region between *fur* and HP1033, and is predicted to regulate the flagellar motor switch gene

(*fliM*) while IG-524 is predicted to regulate fumarase (*fumC*) (256). Recently, Sharma et al. identified hundreds of predicted sRNA candidates and validated the expression of approximately 60 of these through Northern blot analysis (206). However, the actual role of these sRNAs has not yet been established in *H. pylori*.

It is well documented among many species that Fur regulates its own expression (52-54, 96, 129, 204, 249). In most organisms, Fur autoregulation is mediated by classical iron-bound Fur repression; iron bound Fur binds and represses its own expression (204, 249). However, *H. pylori* Fur autoregulation appears to be much more complex; autoregulation involves both iron-bound Fur repression and *apo*-Fur activation through the use of three Fur binding sites in the *fur* promoter (53, 54). Both iron-bound and *apo*-Fur show equal affinity for the first binding site (-34 to -66) while iron-bound Fur has a higher affinity for the second site (+19 to -13), and *apo*-Fur has a higher affinity for the third (-87 to -104) (53, 54). The equal affinity of both forms of Fur for the first site suggests that it is able to act as an UP element and enhance binding of the RNA polymerase (54). The complexity of *H. pylori fur* autoregulation is perhaps another sign of the importance of Fur regulation.

Given the fact *H. pylori* colonizes the dynamic gastric site, it is perhaps surprising that the *H. pylori* genome is predicted to encode few transcriptional regulators and two component systems (7, 201, 237). Survival of *H. pylori* in the gastric niche in light of the paucity of regulators and two component systems has perhaps led to the acquisition of additional regulatory functions in the regulators that are present. Fur is certainly no exception with the capacity to regulate in its *apo* form and its more complex autoregulation. Additionally, in *H. pylori*, Fur regulates a broader class of genes; Fur

regulates not only genes directly involved in iron uptake and storage but genes involved in acid acclimation (244, 246), nitrogen metabolism (246), and oxidative stress response (55, 66).

Diverse Fur regulon

Acid

Acid tolerance is the ability of bacteria to maintain a near neutral periplasmic pH when exposed to acidic pH. In other bacteria, the alternative sigma factor, RpoS, is an important factor in acid tolerance (130, 217). However, no RpoS homologue has been identified in *H. pylori* (168). In *H. pylori*, the nickel responsive regulator, NikR, directly regulates the expression of Fur and is involved in the response to acid. Additionally, the pH sensing histidine kinase two component system, ArsRS, regulates genes in the Fur and NikR regulons (28, 41, 182, 183). Fur is essential for *H. pylori* growth at low pH (21) and, perhaps not surprisingly, has been shown to regulate an expanded number of genes when exposed to low pH *in vivo* and *in vitro* (83). The chronic upregulation of acid acclimation genes observed *in vivo*, many of which are regulated by Fur, is in keeping with habitual exposure of *H. pylori* to low pH *in vivo* and implicates a role for Fur in colonization.

Oxidative stress

While oxygen is required for *H. pylori* growth, oxygen in combination with divalent metals (i.e. iron, zinc, copper) can generate superoxides and hydroxyl radicals that can result in DNA damage and cell death (157, 239). Oxidative stress is not only

derived from the oxidation of metals, but can also originate from the host's attempt to eradicate *H. pylori* (167). Perhaps not surprisingly, Fur has been shown to play a role in oxidative stress in several organisms (101, 107). In *H. pylori*, superoxide dismutase (*sodB*) helps protect the bacterium from oxygen radicals and is directly repressed by *apo*-Fur (66). Another example of a Fur regulated gene involved in oxidative stress is the neutrophil activating protein, NapA. NapA binds to iron *in vitro* and protects *H. pylori* from oxidative stress (42, 155, 176, 238).

Fur and virulence

In addition to regulating genes involved in iron regulation, Fur is capable of regulating virulence genes in many organisms. In *P. aeruginosa*, Fur appears to regulate genes involved in biofilm formation and toxin production (13, 174, 185) while in *V. cholerae*, Fur regulates the production of hemolysin (225). In *N. meningiditis*, Fur regulates genes involved in toxin production, multidrug resistance, and adhesion (91). In *E. coli*, Fur regulates the Shiga toxins (32), fimbrial adhesion (118), and hemolysin (80) while in *H. pylori*, Fur indirectly regulates the vacuolating toxin, *vacA* (83). Given the intricate role of Fur in this diverse group of bacteria, it is clearly evident that Fur plays an important role in the virulence mechanisms among many bacterial pathogens.

In *H. pylori*, Fur has been shown to be important in colonization of both gerbils and mice (28, 83). In mice, the *H. pylori fur* mutant shows a 2 log difference in the number of bacteria recovered one month post infection (28). Previous colonization studies in Mongolian gerbils have shown that the *H. pylori fur* mutant exhibits decreased bacterial load early in colonization but achieves wild type levels at later stages of infection (83). Additionally, the *fur* mutant is outcompeted during co-colonization assays

with wild type *H. pylori* (83). Even though Fur is found in a broad spectrum of bacteria, the role of Fur over the course of infection and which Fur regulated genes are required for the establishment of infection and development of pathology are not clearly understood. Thus, there is still much to be learned from these animal models of *H. pylori* infection and pathogenesis.

H. pylori *animal models*

Despite the fact that *H. pylori* successfully colonizes over half of the world's human population, many questions remain as to the factors which regulate the development of disease. These include specifics on natural transmission of *H. pylori*, host-pathogen interactions and how the bacterium colonizes the gastric mucosa. Human studies are limited by many factors. Included among these are (1) only a few biopsy samples can be taken during the course of an endoscopy, (2) the endoscopy biopsy techniques are unable to preserve the natural architecture and distribution of the mucosal layer, (3) the development of pathologies like gastric cancer are rare, (4) the genetic diversity of humans complicates attempts to study host factors, and (5) due to the carcinogenic nature of *H. pylori*, once an infection is detected it must be eradicated for ethical reasons.

Despite these limitations, human studies have not been for naught. For example, examination of individuals with a family medical history of gastric cancer revealed that polymorphisms in the gene cluster around IL-1β, a proinflammatory cytokine that inhibits secretion of gastric acid, results in an increased risk of gastric cancer (62). Specific pathologic changes in the human gastric mucosa from *H. pylori* infection have

also been documented (108). While patient samples and data are used for epidemiological data and other studies, in the face of the aforementioned constraints, the development of animal models has been actively pursued.

Chimpanzees (102), cynomolgus (71), rhesus (71), and Japanese monkeys (210) have all been reported to be successfully colonized with *H. pylori*. Just as humans are suspected to acquire *H. pylori* early in life, rhesus macaques are able to naturally acquire *H. pylori* infection as newborns (220). Perhaps not surprisingly, the gastric lesions induced in these models are similar to humans (82). The relatedness of these animals to humans has allowed for utilization of these models to study the effects of carcinogens and heredity on *H. pylori* infection. From this work we have learned about the synergistic effect of dietary nitrosamines and *H. pylori* infection on the development of gastric cancer (134), the acute immune response to *H. pylori* infection (100, 149), and the role of host genetics and *H. pylori* strain differences in host susceptibility to infection (57, 58). Even though nonhuman primates have the benefit of closely related anatomical and physiological features to humans and long life spans, the long period between infection and disease development is a serious barrier for their use (82). Additionally, they are, unfortunately, not practical for use in most laboratory settings for economic and ethical reasons.

The bacteria we now know as *Helicobacter canis and H. felis* were first identified over a century ago by Bizzozero in the gastric mucosa of dogs (22). Subsequently, his counterpart Salomon developed the first *Helicobacter* animal model (1896) long before the discovery of *H. pylori*, (22, 144, 199). Salomon fed the ground gastric mucosa from infected dogs and cats to his mouse colony and was able to propagate *H. felis* in the mice

(199). In the subsequent years, several *in vivo* models have been developed to varying success. Since Salomon's initial foray into the mouse model there have been several rodent models established to study the effect of *Helicobacter* infection. These small mammals are much more feasible for broader use due to their size, consistent genetic background, and decreased price as compared to the nonhuman primates. Despite the differences between humans and rodents, *H. pylori* modulation of immune response and disease development has been extensively explored in these models.

Table 1 Comparison of Helicobacter *infection in C57BL/6 and BALB/c Mice*

C57BL/6	BALB/c
Th1 responder (158, 161)	Th2 responder (158)
Inflammation <6 months (162)	Minimal inflammation at 22+ months (162)
No adenocarcinoma *with H. pylori* (120, 236, 250)	Development of MALT lymphoma (63, 64)
Adenocarcinoma with *H. felis* (30)	No adenocarcinoma with *H. pylori* or *H. felis* (162, 250)

The most popular mouse models of *H. pylori* infection include infection of BALB/c and C57BL/6 mice (Table 1) with either *H. pylori* or *H. felis*. While *H. felis* is closely related to *H. pylori* (56), there are a few important differences. In contrast to *H. pylori*, *H. felis* is encased by periplasmic fibrils and naturally infects cats and dogs (50). The lipopolysaccharide (LPS) of *H. felis* is proinflammatory while *H. pylori* LPS shows low biological activity and poorly induces inflammatory cytokines (25, 170, 197). Perhaps the most striking distinguishing characteristics are the lack of the *cag* pathogenicity island and the vacuolating toxin *(vacA)* in *H. felis* (162, 255). However, *H. pylori* poorly colonizes mice (128), whereas *H. felis* is able to infect a wider range of mouse strains. *H. felis* has consequently been extensively used as an *H. pylori* surrogate in the mouse model.

Both *H. felis* and *H. pylori* have been used to infect C57BL/6 mice, which are considered Th1 responders (127, 153, 158, 162). *H. pylori* infection of these animals induces severe hyperplasia of the mucosa, and atrophy of the chief and parietal cells. However, disease does not progress to gastric adenocarcinoma (196, 230). Conversely, *H. felis* infection induces gastritis and, 15 months post infection, gastric adenocarcinoma develops. This can be inhibited by eradication of *H. felis* (30, 31). However, while *Helicobacter* infection in mice induces atrophy with histologic features similar to humans, they do not tend to induce the active or chronic gastritis characteristic of human *H. pylori* infection (196).

Accordingly, BALB/c mice, which are considered Th2 responders have also been infected with *H. felis* and *H. pylori* (158). When infected with *H. felis*, BALB/c mice do not develop gastritis for most of their lives. However, older mice can develop chronic

gastritis with follicular lymphoid infiltrates in the corpus that can progress to lymphoma in some animals (64). Despite the chronic gastritis in this model, gastric carcinoma has not been detected (127). However, in this model MALT lymphoma has been reported that shows some of the human histological characteristics (63, 64). Accordingly, akin to humans infected with *H. pylori*, antibiotic treatment of infected BALB/c mice leads to eradication of infection as well as a decrease in MALT lymphoma (63).

These mouse models also have the advantage of host genetic manipulation. *H. felis* infected C57BL/6 mice containing mutations in the tumor suppressor gene *apc*, have less severe inflammation and no accelerated rate in gastric cancer development (77). In contrast, *H. felis* infected C57BL/6 mice with a p53 tumor suppressor mutation show a higher proliferative index despite the fact that none of them develop gastric cancer (78). These small animal studies are helping elucidate the influence of host genetic background on the development of disease in ways that the larger models simply cannot.

The WHO made the decision to classify *H. pylori* as a class I (definite) carcinogen on the basis of epidemiological evidence alone (2). Subsequently, animal models have provided the conclusive link to show that *H. pylori* infection causes the development of gastric cancer (111). The development of gastritis, ulceration, intestinal metaplasia, and gastric carcinoma have been successfully observed in the Mongolian gerbil model post *H. pylori* infection with and without the addition of additional carcinogens (103, 104, 229, 251). Furthermore, the pathology observed in Mongolian gerbils develops analogously and is histologically similar to that in humans (79, 251). For the aforementioned reasons, the Mongolian gerbil model has become one of the most widely used animal models for the study of *H. pylori*.

There are many parallels to human *H. pylori* infection and disease progression observed in the Mongolian gerbil model. For example, in humans, hypergastrinemia is correlated with *H. pylori* infection and is reversed when the infection is eradicated (151). A similar *H. pylori* associated hypergastrinemia is observed in the gerbil model (180, 232). This model has also been useful to study the effect of host genetics. For instance, polymorphisms in IL-1β levels in humans are correlated with increased risk of gastric cancer (62) and IL-1β levels in Mongolian gerbils have been shown to be associated with acid secretion (232). While the timeline of disease progression is accelerated in Mongolian gerbils, as compared to humans, eradication of *H. pylori* can decrease the incidence of gastric carcinomas similar to what is seen in humans (208, 209). While Mongolian gerbils may not be able to exactly reproduce the human-pathogen interaction due to the accelerated course to gastric cancer, this model is extremely useful for the study of the mechanisms of bacterial factors on host pathology.

Direction of study

During the past 30 years, *H. pylori* and Fur have been the subject of intense study. However, gaps in knowledge remain in both of these fields. Despite the fact that Fur has been identified in many Gram positive and Gram negative bacteria, *apo*-Fur regulation has only been definitively identified in *H. pylori*. Likewise, regardless of the importance of Fur in *H. pylori*, its specific role in colonization, infection, and induction of host pathology remains undefined. The thesis work described herein is dedicated to the study of Fur regulation and is divided into two major sections. The first section addresses the ability of other bacterial Fur proteins to complement *apo*-Fur regulation. The second

section provides an in depth characterization of the role of Fur in colonization, infection, and pathology in the Mongolian gerbil model of *H. pylori* infection. Together these studies should provide insight into the mechanism of Fur regulation and the importance of *H. pylori* Fur regulation in *H. pylori* infection and pathology.

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Chapter 2

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 (9). This fact seems remarkable when one considers that this site encounters large fluctuations in pH (36), iron availability (2), and other stresses (44). 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 (10, 16, 24). Included among these is the Ferric Uptake Regulator (Fur), which is involved in *H. pylori* colonization (10, 24) and is a necessary component for adaptation to low pH (8) and iron limitation (7).

In most organisms, iron is essential (43) 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 (25). Thus, there must be a delicate balance between acquiring a sufficient amount of iron but not so much as to overload the system. 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 (27). 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 (20, 24, 50). 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 (17, 18, 21). 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 (20, 24). 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 (18, 21, 46). 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* (30) and *Desulfovibrio vulgaris* Hildenborough (4) 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 (14) 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 conditions

The strains and plasmids used in this study are listed in Table 2. 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 2, 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 (40), *D. vulgaris* Hildenborough NCIMB 8303 (29), *E. coli* O157::H7 EDL933 (41), *P. aeruginosa* PAO1 (47) or *V. cholerae* N16961 (28) 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 (32) 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 3.

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

Table 2 Plasmids and strains used in this study

Plasmid or strain	Description	Reference
Plasmids		
pDSM226	pGEM T-easy :: *H. pylori fur*	(13)
pDSM340	pTM117 :: *H. pylori fur*	(13)
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*	This study
pDSM758	pTM117 :: Hp *D. vulgaris*	This study
H. pylori strains		
DSM300	G27 *Δfur* :: cat, Cm^r	(13)
DSM343	G27 *Δfur* (pDSM340), Kan^r Cm^r	(13)
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

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 3). Each of these *H. pylori fur* promoter – heterologous *fur* coding sequence products was initially subcloned into the pGEMT-Easy vector (Promega, USA) (Table 2) 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* (13). 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 (13). Each of these vectors was next transformed into DSM300, which is a *H. pylori ⊿fur* mutant of strain G27 (24). Transformants were selected on the appropriate antibiotics (Table 2). 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.

Table 3 Primers used in this study

Primer[b]	Sequence (5'-3')[a]	Reference
RPA primers		
amiE-RPA-F	GGTTTGCCTGGGTTGGAT	(24)
amiE-RPA-R	GATTTTGCGGTATTTTG	(24)
pfr-RPA-F	GCGGCTGAAGAATACGAG	(13)
pfr-RPA-R	CTGATCAGCCAAATACAA	(13)
Hpfur RPA F	GAGCGCTTGAGGATGTCTATC	(13)
Hpfur RPA R	GTGATCATGGTGTTCTTTAGC	(13)
Cjfur RPA F	CCTGATTTAAATGTAGGAATTGC	This study
Cjfur RPA R	AAAGCTGCATCAAATGCCCTG	This study
Dvfur RPA F	CAACAGCCTCAAGGTGAC	This study
Dvfur RPA R	GTTCGATGTCGTCGTCGA	This study
Ecfur RPA F	GGAGCCGGACAACCATC	This study
Ecfur RPA R	CGCTTCGATGGAATCATC	This study
Pafur RPA F	GACTCGGCCGAGCAAC	This study
Pafur RPA R	ATTTCCTTCTGGCGCTTCTC	This study
Vcfur RPA F	CTCCCACGGCTTAAGATTTTAG	This study
Vcfur RPA R	GACGTTGTTCAATCACATCG	This study
SOE primers		
FurCF1	TCTAGAAAGGCTCACTCTACCCTATT	(13)
CjfurR (SalI)	GTCGACAAATGAGGATAAGGATTGATCCC	This study
CjSOE F	CATTTTACGGATAAGGGAAATATCAGCATGCTGATAGAAAATGTGGAATATG	This study
CjSOE R	CATCATATTCCACATTTTCTATCAGCATGCTGATATTTCCCTTATCCGTAAAAT	This study
Dvfur R	GGTACCTCGTTCACCCGCAC	This study
DvSOE F	CATTTTACGGATAAGGGAAATATCAGCATGAAGGAACCCATCGCCGTATTTC	This study
DvSOE R	GAAATACGGCGATGGGTTCCTTCATGCTGATATTTCCCTTATCCGTAAAATG	This study
DvSOE F2	GAAGCTCCTGTGCGACTCAGGTCTCGCCAAGGAAGTGC	This study
DvSOE R2	GCACTTCCTTGGCGAGACCTGAGTCGCACAGGAGCTTC	This study
EcFurR (SalI)	GTCGACGATAAGGTCTGGCAGGAAATTCGC	This study
EcSOE F	CATTTTACGGATAAGGGAAATATCAGCATGACTGATAACAATACCGCCCTAA	This study
EcSOE R	CTTTCTTTAGGGCGGTATTGTTATCAGTCATGCTGATATTTCCCTTATCCGTAA	This study
PafurR (KpnI)	GGTACCTGGCCGCCCAGAACTGAAC	This study
PaSOE F	CATTTTACGGATAAGGGAAATATCAGCATGGTTGAAAATAGCGAACTTCGAA	This study
PaSOE R	GCTTTTCGAAGTTCGCTATTTTCAACCATGCTGATATTTCCCTTATCCGTAAAA	This study
VcFurR (SalI)	GTCGACAACCCACCATTCGGTGGG	This study
VcSOE F	CATTTTACGGATAAGGGAAATATCAGCATGTCAGACAATAACCAAGCGCTAA	This study
VcSOE R	CCTTTAGCGCTTGGTTATTGTCTGACATGCTGATATTTCCCTTATCCGTAAAAT	This study

[a]Restriction endonuclease sites are underlined

[b]Important restriction sites are included in parentheses

RNase Protection Assays (RPAs)

Each of the heterologous expression strains, as well as the wild type and *Δfur H. 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 (49). 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 3. To measure iron-bound and *apo*-Fur regulation, riboprobe templates were also generated using the primer pairs listed in Table 3 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 (13). 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, bacterial lysates 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-polyacrylamide gel 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 (12). 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

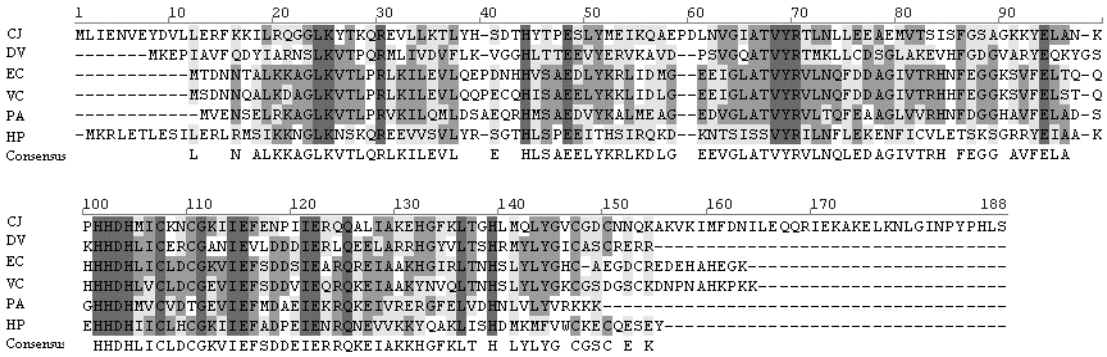
Comparison of the amino acid sequence of Fur from *H. pylori* to Fur encoded by several bacterial species in which Fur has been well studied (4, 30, 42, 45) showed a moderate degree of conservation among the Fur proteins (Table 4, Figure 3). Of note, of the species examined, the highest degrees of identity 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 (4, 30). 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.

Table 4 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 (11).

Figure 3. 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).

Analysis of iron-bound Fur complementation

To determine whether the individual heterologous Fur constructs could complement iron-bound Fur regulation in the *⊿fur* 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 (50). As shown in Figure 4A, 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 *⊿fur* strain (0.6 fold), which additionally shows increased basal level expression of *amiE* even in the presence of iron (Figure 4A). 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 Figure 4B and C. 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 previously noted (13), 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 (Figure 4B). 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 *⊿fur* strain showed an increased basal level expression of *amiE* (3.6 fold) even in the presence of iron (Figure 4A), 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 previously noted (13), 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 (Figure 4C). 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.

Figure 4. Determination of the ability of the heterologous constructs to complement iron-bound Fur regulation of amiE.

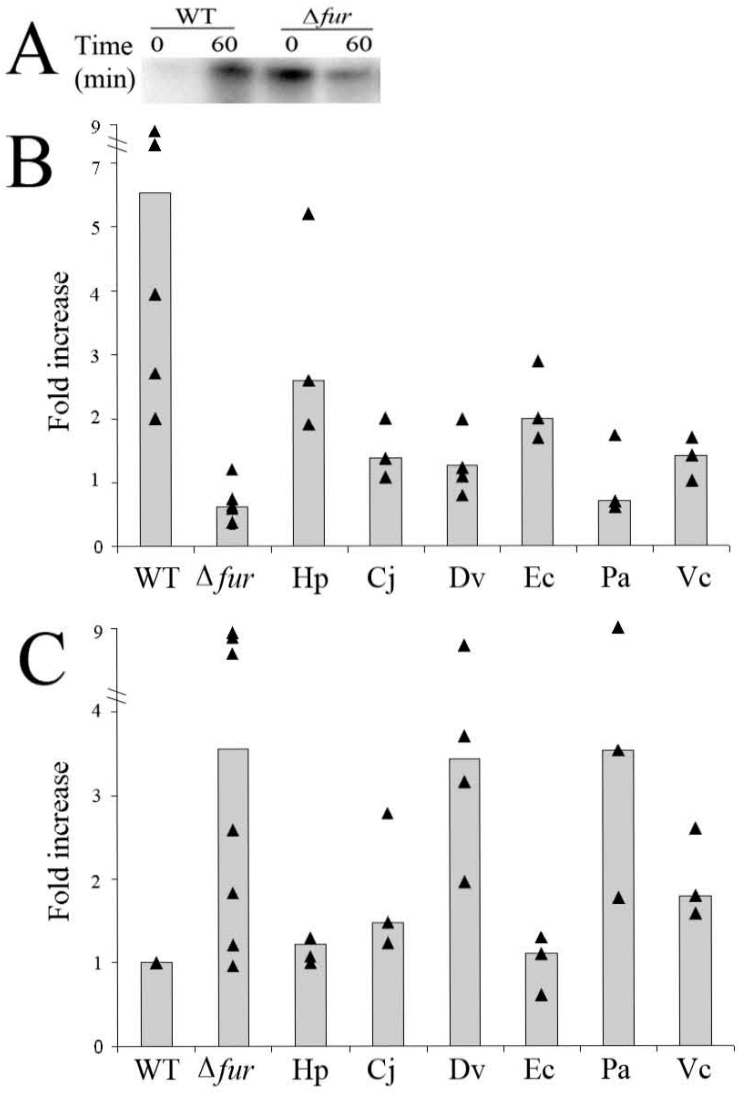
Wild type *H. pylori* (WT), *Δfur H. pylori* (*Δfur*), and *Δfur H. pylori* carrying the heterologous Fur constructs from *C. jejuni* (Cj), *D. vulgaris* (Dv), *E. coli* (Ec), *H. pylori* (Hp), *P. aeruginosa* (Pa), and *V. cholerae* (Vc) were grown to exponential phase in iron replete liquid media. 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 4. Determination of the ability of the heterologous constructs to complement iron-bound Fur regulation of amiE.



Comparison of apo-*Fur complementation.*

Despite the identity and similarity among the Fur proteins (Figure 3, Table 4), *apo*-Fur regulation has thus far only been definitively identified in *H. pylori* (18, 21, 46). 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 (18). As shown in Figure 5A, 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 (18, 21); 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 (13), 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 (Figure 5B). 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 (Figure 5A), 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 (13), 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 (Figure 5C). Analysis of *pfr* basal level expression in the absence of iron in strains carrying the heterologous Fur constructs showed the following changes (Figure 5C): *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.

Figure 5. Determination of the ability of the heterologous constructs to complement apo-Fur *regulation of* pfr.

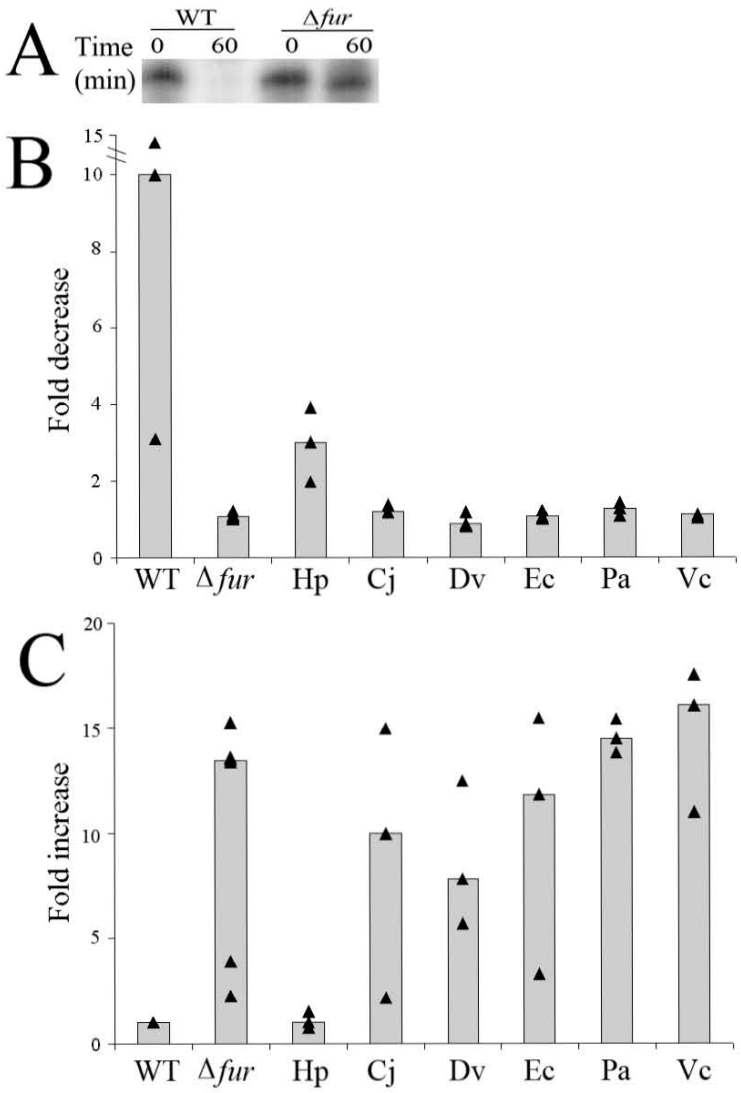
Wild type *H. pylori* (WT), *Δfur H. pylori* (*Δfur*), and *Δfur H. pylori* carrying the heterologous Fur constructs from *C. jejuni* (Cj), *D. vulgaris* (Dv), *E. coli* (Ec), *H. pylori* (Hp), *P. aeruginosa* (Pa), and *V. cholerae* (Vc) were grown to exponential phase in iron replete liquid media. 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 5. Determination of the ability of the heterologous constructs to complement apo-*Fur regulation of* pfr.

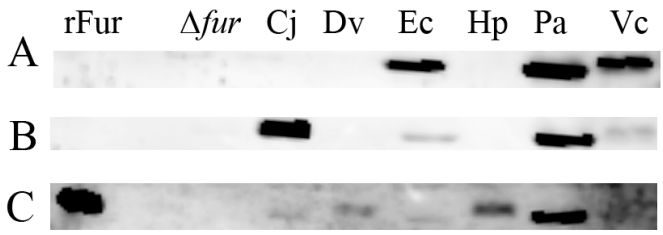


Confirmation of expression and translation of fur *transcript.*

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 3, 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 6A, 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 ΔfurΔ* strain. The *C. jejuni* Fur antibody was able to detect expression of *C. jejuni*, *E. coli*, *P. aeruginosa*, and *V. cholerae* Fur (Figure 6B) 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 (Figure 6C). Taken together, these data indicate that each of the heterologous Fur species is translated and accumulated within the context of the *ΔfurΔ H. pylori* strain (Figure 6). 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 4), there must be considerable Fur structural differences among the various species.

Figure 6. 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.

Discussion

Fur has been characterized in a diverse number of bacterial species, and shown to play a crucial role in iron homeostasis (22, 23, 26, 31, 39, 51). 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 (5, 18, 21). 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 (Figure 4B,C). 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%) (1, 3), 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 (37). Indeed, previous studies have suggested that iron-bound *H. pylori* Fur recognizes a poorly defined conserved A/T-rich consensus Fur box sequence (AATAATNNTNA) (37), which is quite different from the *E. coli* Fur box (GATAATGAT[A/T]ATCATTATC) (15). Interestingly, however, Bereswill, et al. observed that *H. pylori* Fur is able to complement an *E. coli fur* mutant strain (6), and herein we found that *E. coli* Fur provided the most efficient heterologous

complementation in the *H. pylori ⊿fur* strain (Figure 4). 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) (18) 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 (42, 45), 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*) (48). 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 (52), a paucity of general transcriptional regulators, and, to date, only four identified sRNAs (54-56). 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 (33, 35, 53). However, in these organisms, this regulation is mediated by the Fur-regulated sRNA RyhB (34). Similar to *apo*-Fur regulation of *sodB* and *pfr* in *H. pylori* (18, 21), RyhB has been shown to regulate *sodB* and ferritin expression in *E. coli* (19, 34, 35), *P. aeruginosa* (53), and *V. cholerae* (38). However, to date, no RyhB homolog has been identified in *H. pylori* (18). 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 3), 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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Chapter 3

Detailed in vivo *Analysis of the Role of* Helicobacter pylori *Fur in Colonization and Disease*

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The work presented in this chapter is the sole work of S. Miles with the following exceptions: M.B. Piazuelo and M.K. Washington scored the sections for inflammation and pathology, C. Semino-Mora provided scored sections for immunohistochemistry, A. Dubois provided support for immunohistochemistry, R.M. Peek and P. Correa provided assistance for the pathology study, and D. S. Merrell provided technical assistance for the animal work.

Abstract

Helicobacter pylori persistently colonizes the harsh and dynamic environment of the stomach of over half of the world's population and has been identified as a causal agent in a spectrum of pathology that ranges from gastritis to invasive adenocarcinoma. The ferric uptake regulator (Fur) is one of the few regulatory proteins that has been identified in *H. pylori.* Fur regulates genes important for acid acclimation and oxidative stress and has been shown to be important for colonization of *H. pylori* in both murine

and Mongolian gerbil models of infection. To more thoroughly define the role of Fur *in vivo*, we conducted an extensive temporal analysis of the location, competitive ability, and resultant pathology induced by a *Δfur* strain as compared to its parental wild type in the Mongolian gerbil model of infection. We found that at the earliest time points post infection, significantly more *Δfur* bacteria were recovered. However, this trend was reversed by day three when we observed significantly increased recovery of the wild type strain. Increased recovery of *Δfur* one day post infection reflected increased recovery from both the corpus and antrum of the stomach. When the wild type strain was allowed to colonize first, the *Δfur* strain was unable to compete for colonization at any point post infection. However, when the *Δfur* strain was allowed to colonize first, wild type efficiently outcompeted the *Δfur* strain only at early points post infection. Finally, we show that the *Δfur* strain shows a delay in the development and severity of inflammation and pathology within the gastric mucosa even after comparable levels of colonization are established. Taken together, these data indicate that *H. pylori* Fur is most important at early stages of infection and illustrate the importance of the ability of *H. pylori* to adapt to its constantly fluctuating environment in establishing infection, inflammation, and disease.

Introduction

H. pylori persistently colonizes the gastric mucosa of over half the world's population (9) and has been classified as a class I carcinogen by the World Health Organization (1) due to its close association with the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. Fortunately, the majority of individuals infected with *H. pylori* only manifest subclinical

gastritis. However, *H. pylori* has also been associated with gastric ulcers and duodenal ulcers in addition to the aforementioned cancers (14). Thus, *H. pylori* infection is the major risk factor for the development of a broad spectrum of gastric diseases (19).

Given the harsh environment of the stomach, it seems remarkable that *H. pylori* is able to persistently colonize the gastric mucosa. In this dynamic gastric niche, *H. pylori* encounters fluctuations in pH and availability of iron and other nutrients. Thus, the ability of *H. pylori* to survive in the face of this tumultuous environment undoubtedly requires adaptive mechanisms that allow the bacterium to alter gene expression in response to changing environmental cues. Despite this need, *H. pylori* encodes a paucity of transcriptional regulators and two component systems (52). Among the identified regulatory factors, the Ferric Uptake Regulator (Fur) has been shown to be necessary for adaptation to iron limitation (8), low pH (7), and oxidative stress (16, 21). In light of the small number of regulators, it is perhaps not surprising that Fur plays such a diverse role in this pathogen.

Classically, Fur functions as a transcriptional repressor such that when iron is available, Fur binds to its ferrous cofactor and represses gene transcription. This classical regulation is used to control expression of multiple genes in *H. pylori* including *amiE* and *frpB1*, which have been implicated in acid tolerance and iron uptake respectively (25). Uniquely, *H. pylori* Fur is also capable of *apo*-regulation such that in the absence of iron, *apo*-Fur can repress its target genes. *sodB*, a superoxide dismutase, and *pfr*, an iron storage molecule, have been shown to be regulated in this manner (16, 21).

While not essential, Fur has been shown to also play a role in colonization of *H. pylori* (13, 25). In mice, there is a two log difference in the number of *fur* mutant

bacteria recovered one month post infection as compared to the wild type strain (13). Conversely, in the Mongolian gerbil model, our group previously showed that an *H. pylori Δfur* mutant displayed a lag in colonization fitness as compared to wild type; the *Δfur* strain displayed a 50 fold decrease in the number of bacteria recovered three days post infection but reached comparable levels of colonization by 14 days post infection. Additionally, we found that when *Δfur* was coinfected with the wild type in competition assays, the mutant displayed a 100 fold early competitive defect that was maintained throughout later stages of infection (25). Taken together, these data suggest that *fur* plays a role in the early stages of colonization in the Mongolian gerbil, which is a robust small animal model to study development of *H. pylori* induced carcinoma (24, 56).

Since Fur is able to regulate an array of genes important for *H. pylori* stress response and has an effect on colonization, we sought to better define the specific role played by Fur *in vivo* during colonization of the Mongolian gerbil stomach. As suggested by previous data, we found that Fur is most important during early points of infection. Examination of the colonization distribution of the wild type and *Δfur* strains within the stomach showed that the *Δfur* strain indiscriminately colonized both the corpus and antrum, but was rapidly cleared from the acid producing corpus of the stomach. Moreover, the surviving *Δfur* bacteria experienced a lag in their ability to grow within the antral region. Finally, we observed an attenuation in the development and severity of host pathology induced by the *Δfur* strain.

Materials and Methods

Bacterial strains and growth

The gerbil adapted *H. pylori* strain 7.13 (24) and the isogenic *fur* mutant DSM143 (25) 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 (HBA) plates which contained 4% Columbia agar, 5% defribrinated horse blood (HemoStat Laboratories, Dixon, CA), 0.2% β-cyclodextrin (Sigma), 10 μg/ml vancomycin, 5 μg/ml cefsulodin (Sigma), 2.5 U/ml polymyxin B (Sigma), 5 μg/ml trimethoprim (Sigma), and 8 μg/ml amphotericin B. *H. pylori* liquid cultures were grown in brucella broth supplemented with 10% fetal bovine serum and 10 μg/ml vancomycin with shaking at 100 rpm. Microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) were generated by an Anoxomat gas evacuation and replacement system (Spiral Biotech) in gas evacuation jars. All *H. pylori* plate and liquid cultures were grown in microaerophilic conditions at 37°C. Where appropriate, cultures and plates were supplemented with 25 μg/ml kanamycin (Kan).

Single strain and competitive animal infections

Six to twelve week old male Mongolian gerbils (Charles River Laboratories International, Inc., Wilmington MA) were fasted for 12 hours prior to infection. Animals were then orogastrically infected with approximately 10^9 *H. pylori*. Two independent biological repeats of each single infection experiment were conducted as follows: for single strain infections, gerbils were infected with either the wild type or *Δfur* strain and

then sacrificed at 1, 3, 5, 8, 14, or 30 days post infection (n=9-10 animals for each group at each point) or 2, 4, 8, or 16 weeks post infection (n=12-14 animals per group at each point). As a control for the 2, 4, 8, and 16 week single infection time course we also included a mock infected control group (n=8-9 animals per time point). At each indicated point, the glandular portion of the stomach was bisected. Half of the tissue was weighed and homogenized in brucella broth with a mechanical homogenizer, and the number of viable CFU counts were enumerated by plating on HBA plates. The remaining half of the stomach was paraffin embedded, sectioned, and stained with hematoxylin and eosin. Sections were then scored blindly by a pathologist for acute and chronic inflammation on a scale of 0-3 corresponding to normal, mild, moderate, or marked inflammation according to the updated Sydney System (17). Since the acute and chronic inflammation scores were similar, they were averaged to produce a single combined score, which is presented in the manuscript. The sections were also scored blindly for the development of dysplasia and invasive adenocarcinoma by two pathologists. Dysplasia and invasive adenocarcinoma were diagnosed according to guidelines previously published (11). Dysplastic mucosa consisted of elongated or branched, irregular glands, with enlarged, crowded cell nuclei that appear stratified. Invasive adenocarcinoma was defined as tubular or irregular structures composed of dysplastic epithelium that penetrated through the muscularis mucosa infiltrating the submucosa.

For coinfections, animals were infected with a 1:1 mixture of wild type and *Δfur* bacteria, representing an input ratio of approximately 10^9 total CFU. Animals were then sacrificed at days 1 and 3 post infection, the glandular portions of the stomachs excised,

weighed, mechanically homogenized, and plated on HBA plates and HBA plates supplemented with kanamycin. The relative number of wild type bacteria was determined by subtraction of the number of kanamycin resistant *Δfur* colonies from the total number of colonies grown on plain HBA plates. Competitive indexes were calculated by division of the number of *Δfur* bacteria by the number of wild type bacteria, followed by corrections for actual input ratios of each strain. Two independent biological repeats of the coinfection experiment were completed yielding a total of n=15 animals for each day examined.

Superinfections with wild type and Δfur *strains*

Gerbils were infected with approximately 10^9 *Δfur* bacteria and then subsequently superinfected with approximately 10^9 wild type bacteria at 1, 3, 7, 14, or 28 days post initial infection. Three independent biological experiments were conducted and yielded n=10-14 animals per time point. Additionally, we conducted a reciprocal study where the initial infection was conducted with wild type bacteria and superinfection was conducted with the *Δfur* strain (n=5 animals per time point). In each case, animals were sacrificed 2 weeks after the second *H. pylori* dosing, and the glandular stomach was excised, weighed, mechanically homogenized, and plated on HBA plates and HBA plates supplemented with kanamycin to determine the relative numbers of wild type and *Δfur* bacteria recovered.

Localization of H. pylori *distribution in corpus, antrum, and mucus of stomach*

To determine the gross distribution of bacteria within the stomach, animals were infected with approximately 10^9 wild type or *Δfur* bacteria. Animals were sacrificed at 1, 3, 5, 8, 14, or 30 days post infection and the glandular stomachs excised. The presented data are from two independent biological experiments with a total of n=10 animals per group at each indicated point. The stomach was splayed and the corpus and antrum were then divided based on gross anatomical features (33). Each section was then weighed, mechanically homogenized, and plated on HBA to determine CFU. To ascertain the distribution of *H. pylori* within the mucus layer or more intimately attached to the stomach tissue, animals infected with approximately 10^9 wild type or *Δfur* bacteria were sacrificed at days 1, 3, 5, 8, 14, or 30 days post infection. At this point, the glandular stomachs were excised, weighed, repeatedly flushed with 1 ml of a 2.5% solution of N-acetyl cysteine (NAC), and then weighed again. The mucus was recovered and plated on HBA and the stomach tissue was mechanically homogenized and plated on HBA to determine CFU counts. The data presented are from two independent biological experiments with a total of n=10 animals per group at each time point.

Statistical analysis

Microsoft Office Excel 2003 was used for $\log_{10}$ two tailed Student's t-tests analysis of colonization data and the two tailed Student's t-tests were used for analysis of

the inflammation data. Fisher's exact test was used for analysis of pathology data (GraphPad 2005).

Results

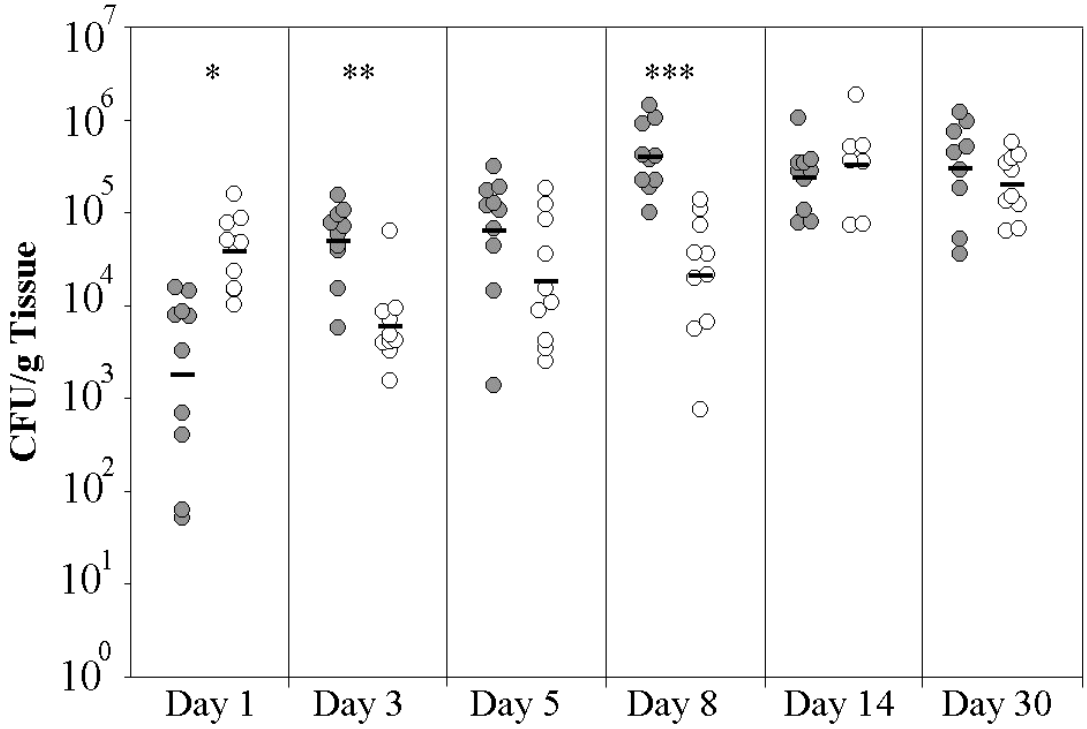
Role of Fur during early points of colonization

Gancz et al. previously showed that at three days post infection, the *Δfur H. pylori* strain achieves a lower colonization density as compared to the wild type 7.13 strain (25). However, by 2 weeks post infection, there is no perceptible colonization difference between the strains (25). This suggests that Fur is important in the early stages of colonization but is nonessential at later stages of infection. To further expand on this initial observation, we performed biological repeats of single strain infections using a larger number of animals and adding additional time points that were not examined in the initial study. We monitored colonization of the wild type and *Δfur* strains at 1, 3, 5, 8, 14, and 30 days post infection. Similar to the previous results, starting at day 3 we observed a reproducible decrease in the amount of *Δfur* recovered as compared to the wild type (Figure *7*). This difference was statistically significant at 3 (p=0.0002), and 8 (p<0.0001) days post infection. Also similar to the previous report (25), this difference gradually decreased over time until we observed no difference in the colonization of wild type and *Δfur* strains starting at day 14 (

Figure *7*). Since Fur appeared to have an effect on early stages of colonization, we examined the effect of Fur at an even earlier point than previously assessed. Unexpectedly, at day 1 post infection, we observed a statistically significant increase in the number of *Δfur* bacteria recovered as compared to wild type (p=0.0007) (

Figure *7*).

Figure 7. Role of Fur in colonization.



Mongolian gerbils were infected with either wild type (grey circles) or *Δfur* (white circles) *H. pylori*. The total colonization of the stomach was determined by sacrificing animals at the indicated time points and is presented as CFU/g of stomach tissue. There was a significant difference in recovery of wild type and *Δfur* at days 1 (**p*=0.0007), 3 (***p*=0.0002), and 8 (****p*<0.0001). Each circle denotes a single animal. Presented data represent two biologically independent experiments and represent a total of n=9-10 animals per group. The geometric mean is represented by the black bar.

Gancz et al. previously showed that when gerbils were infected simultaneously with wild type and *Δfur* strains, there was a 100 fold defect in the recovery of *Δfur* at days 6 and 20 post infection (25). Given the unexpected increase in the *Δfur* strain recovered at day 1 as compared to day 3, we wondered whether a similar competitive defect would be observed at earlier time points. Thus, to examine earlier points in colonization, we coinfected animals with wild type and *Δfur* and monitored the colonization at days 1 and 3 post infection. At day 1 we observed a CI of 0.114, which represents a 10-fold defect in competition for the *Δfur* strain. Similar to the previous study, the CI at day 3 was 0.012, which represents a 100-fold defect. The 10-fold increase in the level of the competitive defect from day 1 to day 3 by the *Δfur* strain suggests that the dynamics of infection change during the initial stages of colonization. Additionally, despite the fact that more *Δfur* bacteria were recovered at day 1 in the single strain colonization data, our coinfection assays suggest that the increased recovery does not imply increased fitness of the *Δfur* strain at this time point.

Role of Fur in establishment and maintenance of infection

Fur appears to be most crucial during the early stages of establishment of persistent infection, but nonessential for maintenance of infection after normal levels of colonization have been achieved. We therefore reasoned that if the *Δfur* strain was allowed to infect first and animals were subsequently superinfected with the wild type strain at various times post infection, we should observe temporal differences in the ability of the wild type strain to displace *Δfur*. The groups of animals were inoculated with *Δfur* bacteria and then superinfected with the wild type strain at 1, 3, 7, 14, or 28

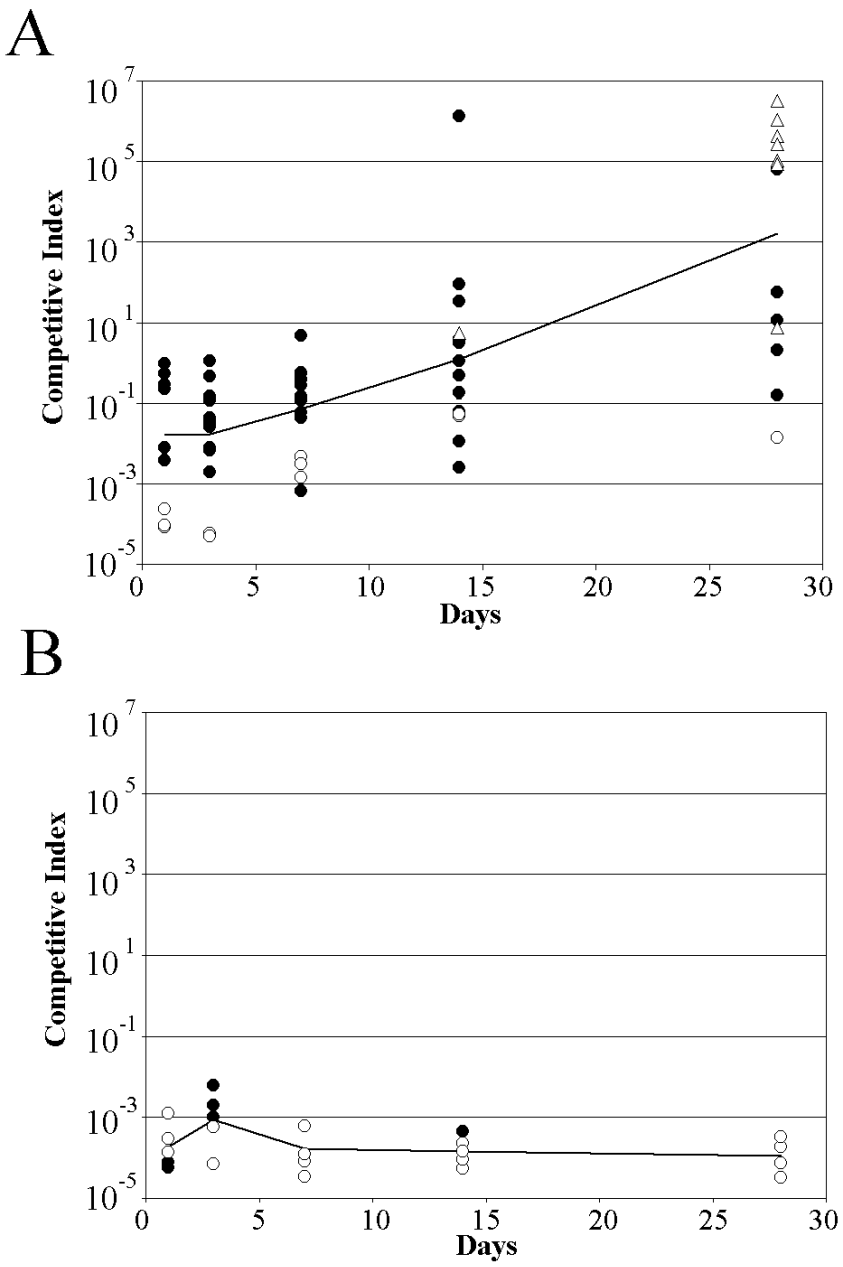
days post initial infection. Two weeks after superinfection, animals were sacrificed and the number of each colonizing strain was determined by plating and the data were expressed as a competitive index (CI) as described in the Materials and Methods. As shown in Figure 9A, the wild type strain was able to displace *Δfur* during the first week of infection; we observed a 100 fold defect in *Δfur* bacteria recovered at days 1 and 3, and a 10-fold defect at Day 7 (Figure 9A). However, the ability of wild type bacteria to displace the *Δfur* strain progressively diminished over time; by day 28, there were no wild type bacteria recovered from 8 of the 14 gerbils. To ensure that this phenomenon was not simply due to colonization dynamics within this model, we also conducted the converse experiment. For this, wild type bacteria were allowed to colonize first and then the animals were superinfected with the *Δfur* strain. We found that *Δfur* was unable to displace wild type at any point (Figure 9B). Taken together, our results indicate that Fur plays a role in establishing infection, but that once colonization has been established, Fur is no longer crucial for maintenance of the infection.

Figure 9. Fur confers an advantage in establishing H. pylori *infection.*

A. Mongolian gerbils were infected with the *Δfur* strain and then subsequently superinfected with wild type bacteria at the indicated time points. Two weeks after superinfection, stomachs were harvested and colonization is presented as CFU/g of stomach tissue. The points represent data from three biologically independent experiments with a combined n=10-15 animals per group at each time point. Black circles represent animals where both wild type and *Δfur* were recovered; white circles represent animals where no *Δfur* bacteria were recovered; white triangles represent animals where no wild type bacteria were recovered. The line denotes the geometric mean across the various time points.

B. Gerbils were initially infected with wild type and subsequently superinfected with the *Δfur* strain at the indicated times. Two weeks after superinfection, the stomachs were harvested and the CFU/g of stomach tissue was calculated. Black circles represent individual animals where both wild type and *Δfur* bacteria were recovered while white circles denote animals where no *Δfur* was recovered. The line denotes the geometric mean across the various time points.

Figure 9. Fur confers an advantage in establishing H. pylori *infection.*



Topography of infection

H. pylori colonization is typically more sparse in the corpus as compared to the antrum (6, 27, 49, 50); however alterations in this distribution have been associated with both host (3, 49) and bacterial factors (51). Given the increased recovery of *Δfur* that we observed one day post infection (

Figure *7*) and the fact that mutation of other *H. pylori* genes have been shown to alter localization of bacteria within the gastric niche (4) we wondered whether Fur was important for localization within the stomach. Initially, immunohistochemical staining of our paraffin embedded sections was used in an attempt to quantitate differences in localization. However, the number of bacteria visible per field in our sections was very low and no overall difference was observed between wild type and *Δfur* in this analysis (data not shown). We reasoned that this incongruence could be due to the combination of the small number of *H. pylori* enumerated in each section and the loss of the mucosal layer during tissue preparation and sample processing. Therefore, to more specifically examine the distribution of *H. pylori* associated with the mucosal layer and those intimately adherent to the stomach mucosa, we separated the mucus from the tissue layers as described in the Materials and Methods and enumerated the number of each colonizing strain found in each fraction. In parallel to the global stomach colonization data (

Figure *7*), at day 1 post infection there was increased recovery of *Δfur* bacteria from the mucus layer (p=0.048) (Figure 10A). By day 3 there was a 10 fold increase in the number of wild type bacteria found in the mucus layer but little overall change in the number of *Δfur* bacteria recovered at this site. This resulted in comparable numbers of wild type and *Δfur* bacteria at this point. Thereafter, the number of wild type bacteria

found in the mucus layer continued to increase until peak recovery was established by day 8. Conversely, the *Δfur* strain showed a decrease in colonization at day 5 which was followed by a much slower increase in the number of recovered bacteria, which peaked at day 14 (Figure 10A).

Analysis of the number of bacteria intimately adherent to the stomach tissue showed that while more of the *Δfur* bacteria were found at day 1, the number of wild type bacteria found adhering to the tissue by day 3 dramatically increased (p=0.003). Thereafter more wild type bacteria were attached to the tissue (day 5 p=0.045, day 14 p=0.006) (Figure 10B). Overall, we observed rapid growth of the wild type bacteria in both the mucosal and stomach tissue layers (Figure 10A, B). Conversely, the *Δfur* strain showed a slight decrease in total colonization from day 1 to day 3 and a lag before growth was initiated (

Figure *7*, Figure 10A,B). Taken together, these data suggest that the *Δfur* strain shows differences in the colonization pattern within the stomach and that Fur is important in initiating growth of *H. pylori* within the gastric niche.

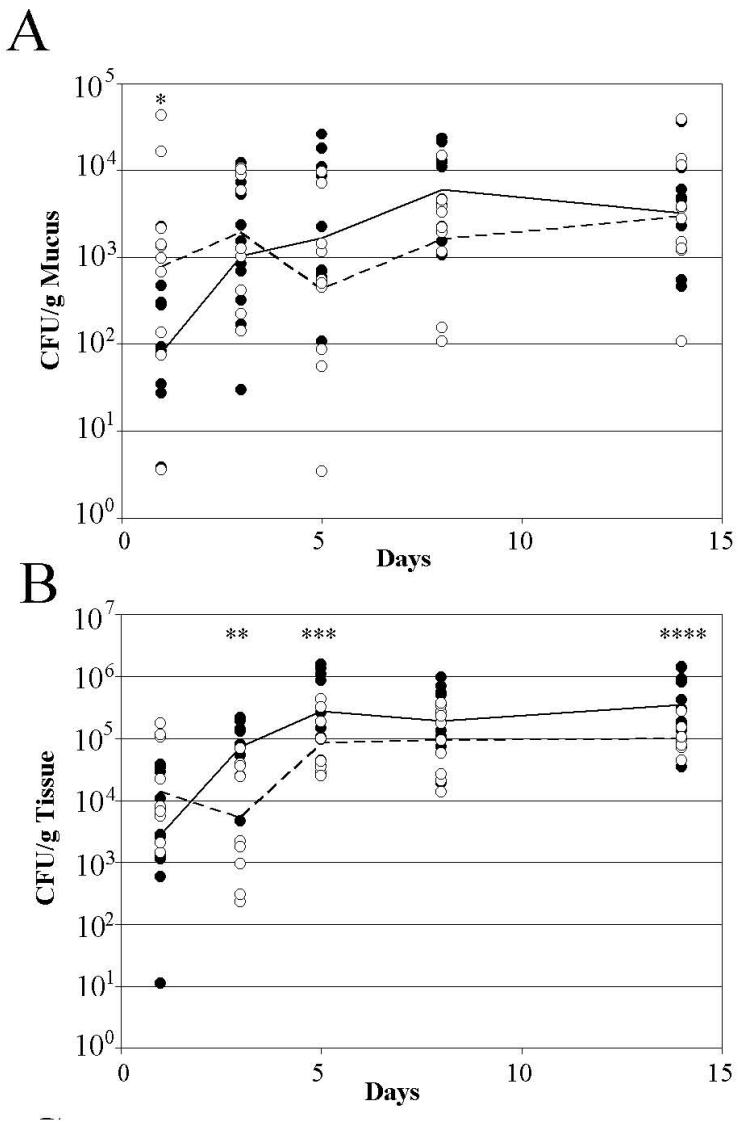
Figure 10. Distribution of H. pylori *in the mucus layer and stomach tissue*

Mongolian gerbils were infected with either wild type or *Δfur H. pylori*. The total colonization of the stomach was determined by sacrificing animals at the indicated time points and is presented as CFU/g of stomach tissue.

A. In an effort to determine the distribution of wild type and *Δfur* bacteria throughout the stomach, harvested stomachs were infused with 2.5% N-acetyl cysteine to separate the mucus from the gastric tissue. The data presented is the combination of two biological experiments with a total of n=10 animals for each group per time point. The data depict the CFU/g of wild type bacteria (black circles) and *Δfur* bacteria (white circles) found within the mucus layer. Each circle denotes a single animal. The geometric mean for the wild type (solid line) and *Δfur* (broken line) strains is denoted. There was increased recovery of *Δfur* bacteria from the mucus layer (**p*=0.048).

B. The CFU/g of wild type bacteria (black circles) and *Δfur* bacteria (white circles) intimately attached to the stomach tissue. Each circle denotes a single animal. The geometric mean across the time points for wild type (solid line) and *Δfur* (broken line) is denoted. At days 3 (***p*=0.003) and 5 (****p*=0.045), and 14 (*****p*=0.006) post infection there were significantly more wild type bacteria recovered from the stomach tissue.

Figure 10. Distribution of H. pylori *in the mucus layer and stomach tissue*

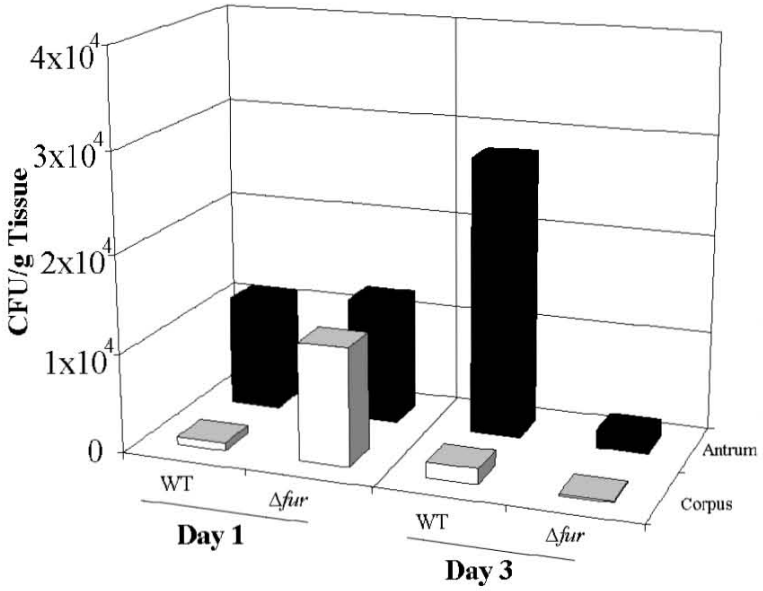


Since Fur regulates a diverse repertoire of genes (20, 25) and because we observed differences in mucosal and adherence distributions, we also wondered if there was a difference in the distribution of wild type and *Δfur* between the corpus and antrum. Thus, animals were infected with wild type or *Δfur* bacteria for 1 or 3 days and the corpus and antrum were separated and examined for colonization as detailed in the Materials and methods. As expected (5), at day 1 post infection the majority of wild type bacteria were localized to the antrum and few bacteria were present in the corpus (Figure 12). This was in stark contrast to the distribution found for the *Δfur* strain recovered 1 day post infection; we observed comparable distribution of the *Δfur* strain throughout the corpus and antrum (10^4 bacteria in each section) (Figure 12). By day 3 post infection there was a dramatic increase in the number of wild type bacteria found in the antrum and a smaller increase in the corpus (Figure 12). Conversely, we observed clearance of the majority of the *Δfur* strain from the corpus as well as a lesser degree of clearance from the antrum (Figure 12). Overall, these findings illustrate the importance of Fur regulation on the ability of *H. pylori* to properly localize to its antral niche where it is able to adapt and colonize.

Figure 12. Distribution of H. pylori *in the corpus and antrum.*

To examine the distribution of *H. pylori* between the corpus and antrum, animals were infected with either wild type or *Δfur H. pylori* and sacrificed at the indicated time points. Stomachs were excised and the corpus and antrum were separated based on gross anatomical features. The colonization of the corpus and antrum at the indicated time points is presented as CFU/g of indicated stomach tissue. The presented data is the combination of three independent biological experiments with a total of n=11-12 animals for each group per time point. The black bars denote the geometric mean of *H. pylori* recovered from the antrum while white bars denote the geometric mean of *H. pylori* recovered from the corpus.

Figure 12. Distribution of H. pylori *in the corpus and antrum.*



Role of Fur induced inflammation and gastric injury

Since Fur plays an important role in regulation of a diverse set of genes and consequently affects colonization, we reasoned that it might also play a role in development of host inflammation and injury. In order to evaluate acute and chronic inflammation, coded sections harvested from animals infected for 2, 4, 8, or 16 weeks were stained with hematoxylin and eosin and blindly graded using the modified Sydney system as detailed in the Materials and methods (17). Since the acute and chronic inflammation scores were similar (data not shown), they were consequently averaged to produce a single combined inflammatory score for each section. These same sections were also blindly examined for dysplasia and carcinoma. For comparison, mock infected animals were dosed with brucella broth and maintained for similar periods of time. No inflammation (Figure 13A, B) or other lesions were observed in any of the control animals (data not shown). We found that even though similar colonization levels of the wild type and *Δfur* strains are achieved by 2 weeks post infection (

Figure *7*), and despite early abundant colonization of the corpus by the *Δfur* strain (Figure 12) there was a statistically significant increase in the degree of inflammation in the corpus of wild type infected animals as compared to those infected with *Δfur* at weeks 4 (p=0.0114), 8 (p=0.0018), and 16 (p= 0.0186); the *Δfur* infected animals never achieved wild type levels of inflammation in the corpus (Figure 13A). Similarly, we observed a lag in the ability of *Δfur* to achieve wild type levels of antral inflammation (Figure 13B). There was more severe inflammation in wild type infected animals as compared to *Δfur* infected animals at week 4 (p=0.0003). However, the *Δfur* strain progressively achieved wild type levels of antral inflammation and was indistinguishable

from the wild type by week 16 (Figure 13B). These observations suggest that while Fur is not required for the development of inflammation, it plays a role in the progression and severity of inflammation.

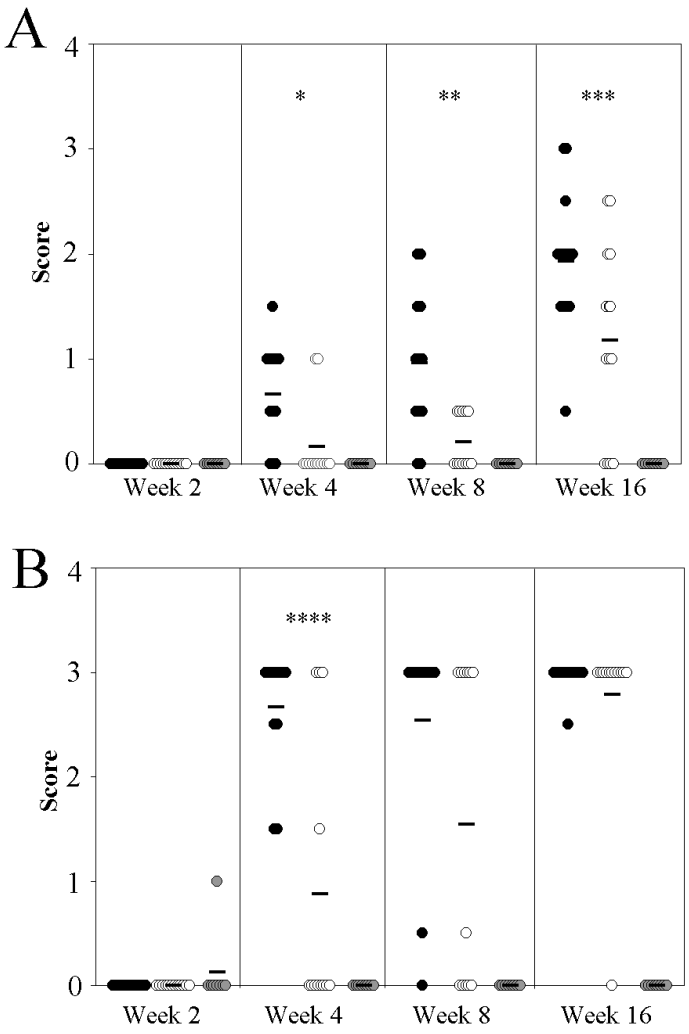
Figure 13. Inflammation scores of the corpus and antrum of Mongolian gerbils.

Mongolian gerbils were mock infected (grey circles) or singly infected with either wild type (black circles) or *Δfur* (white circles) *H. pylori*. Each circle denotes a single animal. At the indicated time, sections of the stomach were fixed, mounted, H&E stained, and coded before blinded scoring for inflammation. Presented data represent two biologically independent experiments and represent a total of n=12-14 animals in each infected group per time point and n=8-9 mock infected animals at each time point. The stomachs were scored for acute and chronic inflammation based on the updated Sydney System (17). Due to their similarity, the acute and chronic inflammatory scores were then averaged to produce a single combined inflammation score for each animal. Mean scores for each time point are denoted by the black bar.

A. The inflammation scores from the corpus of the stomach from two biologically independent infection experiments. There is a significant difference as calculated by the student's t-test, in the degree of inflammation of the corpus between wild type and *Δfur* at weeks 4 (*p=0.0114), 8 (**p=0.0019), and 16 (***p=0.0188).

B. The inflammation scores from the antrum from two biologically independent experiments. There is a significant difference in the degree of inflammation of the antrum between wild type and *Δfur* at 4 weeks post infection (****p=0.0004).

Figure 13. Inflammation scores of the corpus and antrum of Mongolian gerbils



Since the degree of inflammation is considered a risk factor for development of cancer (43), we sought to determine whether there was a parallel delay in the development and severity of premalignant and malignant lesions in Δ*fur* infected animals. Therefore, tissue sections were graded for the appearance of low grade dysplasia and invasive adenocarcinoma as detailed in the Materials and Methods. As expected based on the inflammation scores (Figure 13A,B), by four weeks post infection, there was more severe gastric injury (p=0.0013) in the wild type infected than in the Δ*fur* infected animals (Figure 15A). At 4 weeks post infection mock infected animals showed no pathology (Figure 15B). Of the wild type infected animals 50% developed gastritis, 33% low grade dysplasia, and 17% invasive adenocarcinoma (Figure 15D). Conversely, in the *Δfur* infected animals 25% developed gastritis (Figure 15C) and 8% developed low grade dysplasia by four weeks post infection. Statistical analysis revealed that there was an increased rate of development of gastritis (p=0.0001), dysplasia (p=0.0005), and cancer (p=0.011) in the wild type infected animals in the period from two to four weeks whereas the Δ*fur* infected animals did not display any significant difference in the development of pathology during this same period. Indeed, it was not until 8 weeks post infection that the *Δfur* infected animals displayed a significant difference in the development of carcinoma (p=0.0046). Whereas 93% of wild type infected animals developed invasive carcinoma by 16 weeks post infection, only 64% of Δ*fur* infected animals developed similar pathology by the same time point. Taken together, these data indicate that while Fur is not essential for disease development, it plays a crucial role in the rate of disease development and overall disease severity.

Figure 15. Gastric histopathology in Mongolian gerbils infected with either wild type or Δfur *strain.*

Mongolian gerbils were infected with either wild type or *Δfur H. pylori*. At the indicated time points, sections of the stomach were fixed, mounted, H&E stained, and coded before being blindly scored for injury.

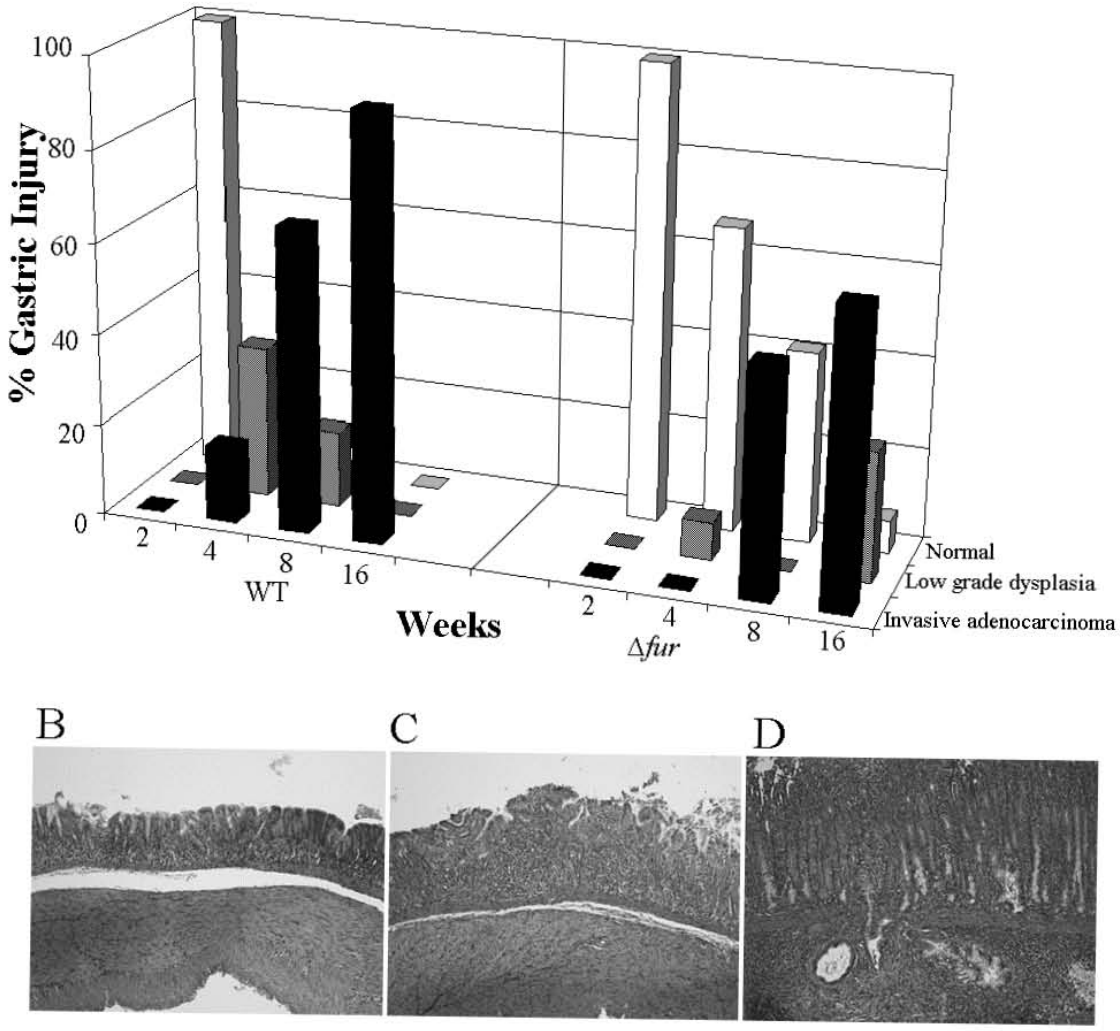
A. Presented data represent two biologically independent experiments and represent a total of n=12-14 animals for each infected group per time point. The data presented are the percentage of animals within each group that showed normal histology (white bars), low grade dysplasia (grey bars), or adenocarcinoma (black bars). While both wild type and *Δfur* infected animals develop adenocarcinoma, there was a delay in the severity and development of injury in *Δfur* infected animals.

B. Normal histology of Mongolian gerbil gastric antrum 4 weeks post mock infection (10X).

C. Gastric antrum from *Δfur* infected animal 4 weeks post infection displaying gastritis (10X).

D. Section from wild type infected animal at 4 weeks post infection displaying adenocarcinoma (10X).

Figure 15. Gastric histopathology in Mongolian gerbils infected with either wild type or Δfur *strain*



Discussion

Pathogenic bacteria must be able to adapt to fluctuations in their *in vivo* environments to successfully survive and colonize. Due to its low pH and overall harsh environment, the stomach was once thought to be a sterile chamber inhospitable to bacterial growth. However, it is now clearly recognized that *H. pylori* is able to successfully colonize this harsh gastric niche. Indeed, it is estimated that over 50% of the world's population is colonized with *H. pylori* (9), which appears remarkable given the dynamic nature of the gastric niche, the constant need to adapt to this fluctuating environment, and the fact that the *H. pylori* genome is predicted to encode a paucity of regulatory factors and two component systems to aid in the process of adaptation (2, 45, 52). Because of this, it has been hypothesized that those regulators encoded by *H. pylori* have acquired additional functions to compensate for the deficit of other adaptive systems. *H. pylori* Fur exemplifies this hypothesis as it regulates genes directly involved in iron uptake and storage (20, 25), as well as genes involved in acid acclimation (54, 55), nitrogen metabolism (55), and oxidative stress response (16, 21). These increased regulatory functions, as well as the diverse *H. pylori* Fur regulon, suggest that Fur plays an important role in *H. pylori* adaptation (37, 54).

We found that despite the fact that initial levels of *Δfur H. pylori* exceeded those of wild type, the *Δfur* strain attempts to colonize an inhospitable site within the stomach and does not properly attach to the stomach tissue. This led to a dramatic reduction in colonization by day 3 post infection (Figure 10). Thereafter, the surviving *Δfur* bacteria displayed a lag in their ability to grow within the antrum but eventually did reach wild type levels of colonization. The gastric mucosa is a dynamic environment where mucus

is constantly secreted from the glands and overall conditions radically change between fed and fasting states (34, 38). Normally, *H. pylori* is found to predominantly colonize the antrum of the human stomach and is believed to primarily reside in the gastric mucus layer (31, 39). Moreover, the distribution of *H. pylori* within the mucus layer is important for maintenance of infection. Indeed, Azevedo-Vethacke et al. previously showed that the administration of proton pump inhibitors, which increase the gastric pH, resulted in the unorganized distribution of *H. pylori* within the mucus layer potentially increasing the possibility that *H. pylori* may be cleared from the stomach (4).

Optimal localization within the stomach requires both motility and chemotaxis, both of which have been shown to be important for colonization of *H. pylori* in numerous animal models (15, 23, 30, 35, 42, 51). Indeed, motility defects due to mutations in the flagellins FlaA and FlaB have been shown to affect *H. pylori* colonization (18, 28). Chemotaxis mutants also show a defect in colonization and persistence or an inability to colonize in certain models (23, 51). Consistent with these observations, *H. pylori* encodes CheW, CheA, and CheY homologues, which affect flagellar direction and velocity, as well as three proteins that are a hybrid of CheW and CheY (CheVs). Unlike other organisms, these CheV proteins do not appear to be redundant with CheW suggesting unique chemotaxic features for these hybrid proteins in *H. pylori*. CheV2 has been suggested by transcriptional profiling to be regulated by *apo*-Fur (20) and is upregulated *in vivo* (46). Additionally, other CheV proteins have been identified to be essential for colonization (29); thus, deregulation of CheV2 in the *fur* mutant may affect chemotaxis abilities that are crucial for initial localization of *H. pylori*. Furthermore, Fur has been shown to regulate expression of the flagellar biosynthesis genes *fliP, flaB,* and

flgE (20). Of these, *flaB* and *flgE* have been shown to be upregulated during gerbil infection (20, 46). In a *fur* mutant, these motility and chemotaxis genes are aberrantly expressed and this fact may be a factor underpinning the ability of the *Δfur* strain to colonize the more proximal corpus of the stomach as well as its inability to reach the antral stomach epithelium to the same extent as the wild type during early stages of infection (Figure 12). Thus, improper localization of the *Δfur* bacteria to the corpus and mucosal layers may, in part, be due to alteration of the expression of multiple motility and chemotaxis genes which require more detailed studies.

Although *H. pylori* colonizes the stomach, it is considered to be a neutrophile and numerous bacterial components aid in its survival within an acidic niche (44). Given the fact that Fur regulation has been shown to be crucial for growth at acidic pH (8), it is perhaps not surprising that genes that are involved in acid acclimation, including the aliphatic amidase, *amiE*, and the formidase, *amiF*, are upregulated *in vivo* (46). These amidases provide alternative sources of ammonia by degrading amide substrates to ammonia and their corresponding organic acid (47, 48). The resulting ammonia then helps to buffer the microenvironment and provide protection for *H. pylori*. Thus, the decreased capabilities of the *Δfur* strain for acid acclimation may be linked to the crucial role of Fur in regulating these factors. Additionally, changes in expression of motility and chemotaxis genes may cause changes in the orientation of *H. pylori* within the mucosal layer which could expose the *Δfur* strain to increased acid stress and could account for the increased clearance of *Δfur* we observed from days 1 to 3 (

Figure *7*). Furthermore, the need to acid acclimate varies within the corpus and antrum of the stomach; the acid secreting corpus has been shown to have a broader pH

range (1.8-4.5) as compared to the antrum (pH 1.6-2.6) (36). While wild type *H. pylori* may be able to sense this gradient and preferentially colonize the relatively low and stable pH of the antral site, the ability of the *Δfur* strain to sense and properly acclimate to the pH gradient is likely compromised. This would account for the dramatic clearance we observe of the *Δfur* strain from the corpus (Figure 12).

Despite the fact that the wild type and Δ*fur* strains achieve comparable levels of colonization by four weeks post infection, we observed a detectable lag in the development and severity of pathology in the *Δfur* infected animals. The fact that both wild type and *Δfur* infected animals developed gastric adenocarcinoma indicates that, while Fur is not essential for disease, it does play a role in the time required to reach this endpoint. Once *Δfur* successfully reaches its proper site of colonization, there is a delay in its ability to evoke host response (Figure 13A,B). One possibility to explain this delay is altered expression of outer membrane proteins (OMPs). It is predicted that 4% of the *H. pylori* genome encodes for OMPs (52), many of which are involved in adhesion (41, 57) and immune modulation (22, 53). Of these OMPs, HopZ is Fur regulated and is involved in adhesion (25, 41). Thus, altered expression of *hopZ* could contribute to the defect in tissue adherence we observed (Figure 10B) as well as subsequent changes in inflammation and injury (Figure 15A). In support of the role of adherence in crosstalk with the host, increased cell adherence of *H. pylori* has been shown to result in an increased host immune response (26). Thus, the observed predilection of wild type bacteria for the gastric tissue versus the mucus layer (Figure 12) suggests that Fur regulated adherence genes such as *hopZ*, may also be involved in the initial colonization of the stomach and the development of inflammation.

An important aspect of *H. pylori* induced disease is the ability of some strains to deliver the virulence factor CagA into host cells. CagA is delivered into epithelial cells through a type IV secretion system and CagA positive strains have been shown to be associated with more severe gastric inflammation and gastric cancer (10, 32, 40). Delivery of CagA and the resulting downstream effects require intimate interaction of *H. pylori* with host cells. Thus, decreased adherence by the *Δfur* strain may decrease the amount of CagA delivered to host cells. Furthermore, the type IV secretion system has also been shown to be important for the delivery of peptidoglycan to host cells, which has been reported to provoke inflammation through Nod1 signaling (12). Thus, in addition to altered CagA delivery, strains displaying altered adherence would provide less host exposure to *H. pylori* peptidoglycan. Taken together, intimate contact between *H. pylori* and gastric epithelial cells is clearly important for host inflammation and disease. Our finding that there are delays in the development and severity of inflammation and gastric injury in our *Δfur* infected animals undoubtedly are a consequence of the multifactorial effects of Fur regulation. These findings also demonstrate that the mere presence or absence of Fur is not responsible for disease. Future studies to examine which Fur regulated genes are important for proper *H. pylori* tissue targeting and colonization of the gastric niche may provide insight into the development of prophylactic therapeutic targets.

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Chapter Four

Discussion

Preface

H. pylori is a neutrophile that must adapt to the dynamic gastric environment to survive and successfully, persistently colonize this harsh site. Genomic studies have revealed that *H. pylori* encodes for surprisingly few regulatory factors and two component systems (63). One of the identified regulators, Fur, has been shown to control a diverse regulon encompassing acid tolerance, nitrogen metabolism, and the namesake iron uptake and storage genes (26, 65). Fur has been extensively characterized in other Gram positive and negative bacteria and shown to act as a repressor in its iron-bound form (5, 32, 34, 35, 47). Despite study in these diverse bacterial species, *H. pylori* Fur has the distinction of being the only Fur protein shown to utilize both iron-bound and *apo*-Fur regulation to control gene expression (17, 24). Fur has also been shown to be involved in colonization and virulence to varying degrees in *H. pylori* as well as other bacterial species (4, 8, 26, 30, 35, 59). The studies described in this thesis were designed to gain insight into the mechanism of Fur regulation and to define the *in vivo* role of *H. pylori* Fur regulation.

In an effort to gain greater insight into Fur regulation, the first section of this work sought to determine whether other bacterial Fur proteins are capable of *apo*-Fur regulation. Using translational fusions of the *H. pylori* Fur promoter to the Fur coding sequence from different bacterial species, we were unable to demonstrate *apo*-Fur regulation with *C. jejuni, E. coli, D. vulgaris, P. aeruginosa,* or *V. cholerae* Fur. The

second section of this work focused on the role of Fur in colonization and virulence in the Mongolian gerbil model of *H. pylori* infection. In this model, we showed that Fur plays a role in the initial distribution and colonization of *H. pylori* within the gastric niche. *H. pylori* Fur was also shown to be nonessential for the development of inflammation and subsequent pathology but crucial for the temporal progression and severity of both.

Discussion and Significance of Findings

Apo-*Fur regulation appears unconserved across bacterial species*

Despite the fact that Fur is well conserved across a diverse range of Gram positive and negative bacteria, to date *apo*-Fur regulation has only been identified in *H. pylori*. To determine whether Fur from other bacterial species is also capable of *apo*-Fur regulation, we constructed translational fusions of the *H. pylori* Fur promoter to the Fur coding sequence from *C. jejuni, E. coli, D. vulgaris*, *P. aeruginosa*, and *V. cholerae* and expressed these proteins within the context of *H. pylori*. We reasoned that if the Fur proteins from these bacteria, which encode a plethora of regulatory proteins with overlapping functions, were put into the context of *H. pylori*, a bacterium lacking extensive regulatory resources, we should be able to determine whether these Fur proteins encode the capacity for *apo*-Fur regulation. In addition, we assessed the ability of these proteins to undertake the classic form of iron-bound regulation within *H. pylori*. We were able to partially complement iron-bound Fur regulation with *C. jejuni, E. coli,* and *V. cholera* Fur. However, even though the examined Fur proteins share a moderate amount of conservation, we were unable to complement iron-bound Fur regulation with

D. vulgaris or *P. aeruginosa.* This data suggests that *H. pylori* Fur has acquired unique structure/function features in comparison to other bacterial Fur proteins.

Despite these findings, we also recognize that it is possible that *D. vulgaris* and *P. aeruginosa* Fur might both be able to regulate some iron-bound Fur genes within *H. pylori* if a different target was chosen for study. We chose *amiE* as it is one of the best characterized Fur regulated genes. However, it is possible that these diverse Fur proteins are unable to recognize and bind the *amiE* DNA target in *H. pylori* due to differences in DNA recognition sequences (Fur boxes). Whereas the Fur box of *E. coli* Fur is highly conserved, the motifs and level of conservation of similar boxes vary among bacteria that encode Fur (2, 15, 27, 43). For instance, the Fur box in *E. coli* consists of three repeats of GATAAT (2), the last of which is inverted with a single nucleotide separating the second and third repeats while the Fur box of *Y. pestis* consists of two inverted repeats of AATGATAAT separated by a single nucleotide (27). The *H. pylori* Fur box consensus sequence is significantly different from the *E. coli* consensus sequence and even among the *H. pylori* Fur boxes there appears to be only modest conservation (43). This suggests that there are unique structure/function features that affect the ability of *H. pylori* Fur to recognize and bind its DNA targets.

In a similar vein, the *C. jejuni, D. vulgaris, E. coli, P. aeruginosa,* and *V. cholerae* Fur proteins were completely unable to complement *apo*-Fur regulation within the context of *H. pylori*. Once again we chose *pfr* because it is a well characterized *apo*-Fur regulated gene (11, 23, 26). However, the same caveat exists; the *pfr* promoter may not be recognized by *apo*-Fur of other organisms. Currently there have been only two genes (*pfr* and *sodB*) that have been shown to definitively be regulated by *apo*-Fur (17, 24).

Therefore, alternative candidate genes to be examined are in short supply since the regulation of *sodB* by *apo*-Fur varies between *H. pylori* strains (10). It is also possible that these other organisms have evolved additional regulatory mechanisms to compensate for a lack of *apo*-Fur regulation. For example, in contrast to the *apo*-Fur regulation of *sodB* and *pfr* in *H. pylori*, the Fur-regulated sRNA RyhB has been shown to regulate *sodB* and ferritin in *E. coli*, *P. aeruginosa*, and *V. cholerae* (22, 38, 44, 45, 69). This suggests that these species use sRNAs in place of *apo*-Fur. To date, there have been no RyhB homologs identified in *H. pylori* (57, 71, 72). Thus, evolution of the regulatory functions of Fur appears to have developed within the framework of the host bacteria.

Within different species the types of genes that Fur controls can very broadly. For example, *D. vulgaris* Hildenborough and *Desulfovibrio desulfuricans* both appear to have extended Fur regulons; i.e. classes of genes are regulated in addition to those involved in iron uptake and storage. This is perhaps surprising given that the *Desulfovibrios* are anaerobic and in anaerobic bacteria there is considerably less likelihood of the formation of reactive oxygen species from the Fenton reaction (31). Additionally, iron is expected to be in its more readily accessible ferrous form. Interestingly, although the *Desulfovibrio* are considered strict anaerobes, they encode a superoxide dismutase protein, which typically functions in combating oxidative stress (21, 25). Additionally, anaerobic δ – proteobacteria such as *Desulfotalea, Desulfovibrio, Desulfuromonas,* and *Geobacter* have been shown to encode *fur*, *perR*, and *zur*, three *fur* paralogs. This suggests that despite the anaerobic nature of these bacteria, there is selective pressure to regulate iron homeostasis and oxidative stress response in these organisms (54). Given that multiple *fur* paralogs exist, perhaps they encode complex

regulatory functions. In support of this, microarray analysis of *D. vulgaris* suggested that it may be capable of *apo*-Fur regulation; nine genes were predicted to be repressed by *apo*-Fur (5). Even though *D. vulgaris* is not closely related to *H. pylori*, their Fur proteins share 30.5% identity and 49.3% similarity. However, *D. vulgaris* Fur was unable to complement iron-bound or *apo*-Fur regulation within the context of *H. pylori* suggesting that their DNA targets or overall regulatory capabilities are different. Clearly, much remains to be understood about Fur function and its role as an *apo*-regulator.

In addition, sequence conservation does not necessitate similar or exact functions. For instance, Fur from both *P. aeruginosa* (52) and *V. cholerae* (58) has been crystallized. However, the crystal structures of *V. cholerae* and *P. aeruginosa* Fur show very different orientations of their DNA binding sites. This is despite the fact that the proteins share 51.3% identity and 70.7% similarity (52, 58). Another prime example of this concept can be found in the comparison of *V. cholerae* Fur and the closely related *Vibrio harveyi* Fur. Though the Fur proteins from these organisms share 96% identity, a proposed critical metal binding site in *V. cholerae* Fur appears to be nonessential in *V. harveyi* (62). Even though the Fur proteins from different species are well conserved and have many similar regulatory capabilities and targets in common, small changes in their amino acid sequences clearly result in large differences in protein conformation and function.

Detailed in vivo *Analysis of the Role of* H. pylori *Fur in Colonization and Disease*

Despite the diverse regulon of genes controlled by *H. pylori* Fur, previous work has shown that Fur is not essential (26). However, Fur plays a role in *H. pylori*

colonization in both the mouse and gerbil models of infection (8, 26). Other bacterial Fur proteins have also been shown to play a role in virulence and pathogenesis in a number of diverse animal models. For example, similar to our observed decrease in virulence of the *H. pylori fur* mutant in gerbils, the closely related *C. jejuni* also demonstrated decreased colonization of *fur* mutants in the chicken model (49). However, no research on the effect of *H. pylori* Fur on the localization of *H. pylori* within the stomach or its consequent effect on disease progression in an animal model was performed prior to our work. Indeed, our study is the first demonstration that Fur is important for the distribution of *H. pylori* within the stomach.

Whereas other bacteria rely on acid tolerance and resistance genes for their relatively brief stay in the stomach (74), *H. pylori* persistently colonizes the gastric niche. Thus, it is not surprising that motility and acid-resistance genes are necessary for successful *H. pylori* colonization of the gastric mucosa; the bacteria must be able to survive the low pH of the stomach lumen and the constant shedding of the mucus as well. In human and rodent stomachs, the more acidic antrum is the preferred site of *H. pylori* colonization, but bacteria can also be found to a lesser extent in the corpus. In fact, the acid secreting corpus has been shown to have a broader pH range (1.8-4.5) as compared to the antrum (pH 1.6-2.6) (41). This is thought to be critical as previous work has shown that the distribution of *H. pylori* in the stomach is affected by pH. For example, when acid secretion is suppressed with proton pump inhibitors, there are more *H. pylori* found in the corpus (68). This suggests that pH is a factor in the localization of *H. pylori* to its antral niche. Distribution of *H. pylori* between the antrum and corpus has also been correlated with disease; antral-predominant gastritis is associated with duodenal ulcers,

whereas corpus-predominant gastritis is associated with gastric ulcers, atrophy, intestinal metaplasia, and gastric adenocarcinoma (18, 36).

Due to the importance of *H. pylori* localization and because mutations in *H. pylori* have been shown to affect distribution of *H. pylori* throughout the stomach (70), we decided to investigate whether there were differences in wild type and *Δfur* colonization of the corpus and antrum. We observed that the *H. pylori Δfur* strain attempts to colonize the corpus, a site less amenable to *H. pylori* during infection. We hypothesize that this could be due to the deregulation of genes involved in chemotaxis and motility in the *Δfur* strain (23). After initial colonization, the *Δfur* strain is rapidly cleared from this site resulting in a decreased recovery of the *Δfur* strain as compared to wild type at day 3 post infection. Additionally, the growth kinetics after this point are slower for the *Δfur* strain. However, the *Δfur* strain is able to achieve wild type levels of colonization at two weeks post infection and maintains comparable bacterial density after this point throughout the infection. Despite this, once similar colonization levels of *H. pylori* are achieved there are significantly less pathology in *Δfur* infected animals.

This discordance of colonization density and pathology illustrates the importance of *H. pylori* Fur in the progression of inflammation and disease. Even though *Δfur* infected animals did develop cancer at later time points, this occurred at a much slower rate and the percentage of animals that developed cancer was dramatically decreased. While the reason for this is unclear, within the spectrum of genes predicted to be regulated by *H. pylori* Fur are those encoding outer membrane proteins and factors involved in adherence (23, 26). Deregulation of these genes could decrease the contact of *H. pylori* to host cells, thus decreasing host exposure to the proinflammatory CagA. This

would result in decreased host response and subsequent pathology. Thus, while Fur is not essential for disease development, it does have a role in the progression of disease.

Additional Studies

Search for sRNAs

As discussed, to date, *apo*-Fur regulation has only been definitively identified in *H. pylori* (17, 24). In other bacteria, genes that were once postulated to be controlled by *apo*-Fur regulation have since been shown to be controlled by Fur regulated sRNAs (22, 38, 39, 44, 69). Most bacterial sRNAs control gene transcription by base pairing with complementary sequences (28). For example, in *P. aeruginosa,* Fur is able to regulate the sRNAs *prrF1* and *prrF2,* which indirectly regulate quorum sensing (48). In Chapter 2 of this thesis, Fur regulation of sRNAs was proposed as an alternative mechanism in organisms lacking *apo*-Fur regulation. Despite the fluctuating environment of the gastric niche, *H. pylori* has a limited number of transcriptional regulators and two component systems and only four sRNAs had been identified at the time of publication of that work (63, 72). Of those, NAT-67 and NAT-39 are complementary to the iron-binding protein *ceuE* and the Fur regulated outer membrane protein *frpB,* respectively (72). Recently, as detailed below, Sharma et al. identified hundreds of predicted sRNA candidates *in H. pylori*; of these predicted sRNAs, they validated the expression of 60 sRNAs through Northern blot analysis (57). However, it is currently unclear whether expression of any of these sRNAs is Fur dependent or affects *apo*-Fur regulation. Thus, additional studies should be conducted in an effort to identify and characterize such RNAs.

Previous *H. pylori* studies have employed a bioinformatics approach to search for putative sRNAs in *H. pylori* to limited success (72). However, this approach relies on sRNA characteristics from other bacteria that may not be valid in *H. pylori.* Relying on the fact that most primary mRNAs and sRNAs carry a 5' tri-phosphate group whereas processed ribosomal and transfer RNAs have a 5' mono-phosphate cap, Sharma et al. developed an assay (dRNA-seq) to enrich for primary transcripts (57). They then compared and mapped the sequences from bacterial RNA that has been treated with terminator exonuclase, which specifically degrades the 5'monophosphate RNA, and untreated bacterial RNA (57). This resulted in the prediction of hundreds of sRNA; included among these are predicted sRNAs associated with both iron-bound and *apo*-Fur regulated genes (57). However, before further study, the expression of these sRNAs should be demonstrated conclusively by Northern blot. If expressed, Northern blot analysis could also be used to assay differences in sRNA transcript abundance in both iron-replete and deplete conditions in both wild type and *Δfur* bacteria to identify candidate Fur regulated sRNAs. Once confirmed, the possibilities for study are numerous. For instance, primary and secondary targets could be identified using microarrays and RNA harvested from *H. pylori* strains over-expressing the sRNA. In addition, while proteins are uncommon targets of sRNAs (12, 67), the binding of expressed sRNAs to the Fur protein could be examined with mass spectrometry. Once targets are identified, the sRNA in question could be mutated to demonstrate elimination of regulation through measurement of the expression of its target. Clearly there are numerous exciting possibilities for further study.

If none of the predicted sRNAs are shown to be regulated by Fur, the search for Fur regulated sRNAs could be continued through use of the dRNA-seq assay. It is interesting to note that the Sharma et al. study was conducted using RNA from both normal, high iron growth conditions as well as acidic conditions (57). In an effort to identify Fur regulated sRNAs this assay could be conducted under both iron replete and deplete conditions with both wild type *H. pylori* and a *Δfur* derivative. The results of the sequencing analyses could then be investigated for additional putative sRNAs. In an effort to identify sRNAs involved in *apo*-Fur regulation, characterization would start with those that are differentially expressed in iron deplete conditions and that show no change in expression in the *Δfur* strain. These proposed studies should allow for the identification of Fur regulated sRNAs.

Characterize role of apo-*Fur regulation in disease*

Given that we showed a role for Fur in colonization and disease progression and severity *in vivo*, it would be interesting to determine specifically whether iron-bound, *apo* or both forms of Fur regulation are required *in vivo*. Previous work has shown that the *apo*-Fur regulated ferritin, *pfr*, is essential for colonization of the Mongolian gerbil (66). In addition, the superoxide dismutase, *sodB*, which is another gene in the *apo*-Fur regulon, has been shown to be important, though not essential, in the colonization of the mouse stomach (56). The fact that the only two genes definitively known to be regulated by *apo*-Fur have are important for colonization suggests that *apo*-Fur regulation may be crucial *in vivo* (17, 24).

The results from our study on other bacterial Fur proteins suggest that *C. jejuni*, *E. coli*, and *V. cholerae* Fur are able to function as iron-bound Fur regulators, but not *apo*-Fur regulators within the context of *H. pylori*. Given this, perhaps the effect of *apo*-regulation *in vivo* could be examined by expressing *E. coli* Fur in *H. pylori*. The *E. coli* Fur protein gave the highest degree of iron-bound complementation in our study and thus, strains carrying *E. coli* Fur should be able to regulate iron bound Fur target genes but not *apo*-Fur target genes *in vivo*. If *H. pylori* strains carrying *E. coli* Fur were inoculated into gerbils and followed over time, the role of *apo* and iron-bound Fur regulation could be determined. Alternatively, Carpenter et al. recently characterized two *H. pylori* Fur mutants that appear to be involved specifically in *apo*-regulation (9). Mutation of amino acid E90 or H134 to alanine caused decreases in the basal level of expression of the *apo*-Fur regulated *pfr* (9). Further characterization showed that, H134A exhibits nonspecific DNA binding and consequently may affect genes that are not typically Fur regulated (9). However, E90A does not interact with promiscuous targets and thus, the effect of *apo*-Fur regulation on infectious dose, colonization, and disease could be studied through use of *E. coli* and the *H. pylori* strains carrying the E90A Fur mutant protein.

Given that *apo*-Fur regulates *sodB* (17), which has a protective effect against oxidative stress, it is formally possible that the effect of a *Δapo*-Fur strain may only appear at later points of infection when the bacterium is faced with the self-induced host inflammatory response. If the *Δapo*-Fur strain is unable to properly regulate superoxide dismutase or ferritin when faced with the increased release of iron from host cell death, this could have a dramatic effect on bacterial survival. Thus, the relationship of the colonization of the *Δapo*-Fur strain and host inflammation should also be examined. This

could be accomplished by infecting animals with either the wild type or *Δapo*-Fur strain and then examining the stomachs over time for colonization and inflammation. We would expect to see a defect in colonization; however, there may be no noticeable consequence of the *Δapo*-Fur strain in colonization or disease if some of the proposed *apo*-Fur regulon has redundant functions with non-Fur regulated genes. Additionally, some genes may also controlled by another regulator (NikR). However, if colonization and/or disease attenuation is observed in the *Δapo*-Fur strain, the characterization of this smaller predicted regulon would be quite feasible and may elucidate targets for disease prevention.

Investigate H. pylori-*host immune response interactions*

In comparison to other bacteria, *H. pylori* elicits an ineffective immune response, which allows for its persistent colonization of the gastric mucosa. This is in part due to the site of colonization. Normally, the stomach is a location relatively devoid of chemokines and cytokines required to guide leukocytes and lymphocytes (40, 50). However, the dynamics of host immunity change upon *H. pylori* infection and we observe associated leukocyte transmigration (14), accumulation of T cells (3), and the classic lymphoid follicle formation (60). This alteration in host response is evident in that, even in the absence of clinical symptoms, virtually all individuals colonized with *H. pylori* develop gastritis (19). Our studies have shown that Fur has an effect on the progression and severity of inflammation and disease, therefore it follows that Fur may affect the direct interaction between the bacterium and the host. Further studies should examine Fur dependent host-pathogen interactions, which include expression of outer

membrane proteins and adhesion, so that researchers can exploit these interactions to prevent or reverse disease development.

Vaccine development has been proposed as a solution to combat the increasing rate of antimicrobial and multidrug resistant strains of *H. pylori* (16, 20, 29, 42), and as a possible solution to reprogram the host immune response to infection. The immune response that is generated in response to *H. pylori* infection is typically not strong enough to clear the infection or prevent reinfection after successful treatment (37). This ineffective immune response is believed to be due to lipid A modifications in *H. pylori* LPS which make it less immunogenic (46) as well as modifications in the TLR5 recognition site on the bacterial flagellum that prevents recognition by the Toll-like receptors (1). Thus, to improve immunogenicity of an *H. pylori* vaccine, coadministration of the agent with an antigenic stimulant such as a subunit of *Salmonella* flagellin might be necessary.

While the positive associations of *H. pylori* with gastritis, peptic and duodenal ulcers, MALT lymphoma, and gastric cancer are well established, an inverse association with some diseases remains controversial. *H. pylori* infection has been reported to have an inverse relationship with the development of gastroesophageal reflux disease (33), Barrett's esophagus (64), esophageal adenocarcinoma (73), diarrheal diseases (55), active tuberculosis (51), asthma, and other allergic disorders (7, 13, 53). Because of this, a vaccination or therapeutic approach to abrogate *H. pylori* induced host disease instead of bacterial eradication would be preferred. In this scenario, Fur could be an ideal target since it appears to have no direct regulatory effect on *cagA* and allows *H. pylori* to colonize but results in attenuation of disease. In this case, vaccines based on the

generation of an immune response targeting Fur or Fur regulated OMPs could be used as a foundation to prevent disease. A two pronged approach could be used to test this idea. The first would be to initially administer the anti-Fur agent and challenge the vaccinated animals. Both treated and untreated groups would be followed over time to compare bacterial colonization and the development of pathology. Successful agents would prevent pathology. Alternatively, animals colonized with *H. pylori* would be administered the agent and then evaluated for bacterial clearance and development (or reversal) of pathology. In this case, successful candidates would be those that prevented or reversed host pathology.

Over half of the world's population is colonized with *H. pylori* (6). Of those individuals, 15-20% develop gastric or duodenal ulcer disease and approximately 1% develop gastric adenocarcinoma translate to a large global medical burden (61). While we have no way to accurately assess how the temporal distance in disease development observed in gerbils translates to human disease, these models can be used to guide the investigation of bacterial factors in disease to hopefully decrease disease rates. The results of this dissertation have provided the basis for further studies to explore the role of Fur in colonization and disease.

Conclusion

Despite the fact that *H. pylori* colonizes a site that was once considered to be a sterile chamber that was inhospitable to bacterial growth due to fluctuations in pH and nutrients, the bacterium is predicted to encode a paucity of regulators and two component systems (63). In the relatively short time since *H. pylori* was discovered, it has been the

subject of intensive study. From this voluminous work, it has been shown that *H. pylori* has evolved to acquire unique regulatory functions to, not only survive, but persistently colonize the gastric niche. The work discussed in this thesis focused on Fur, a global regulator in *H. pylori*. We have shown that the *apo*-Fur regulation found in *H. pylori* appears unconserved across *C. jejuni, E. coli, D. vulgaris, P. aeruginosa,* and *V. cholerae*. Second, we have shown that within *H. pylori*, Fur plays a role in establishing infection and proper localization to the antral niche. However, while Fur is not crucial for maintenance of infection, it plays a role in progression and severity of inflammation and pathology.

Future experiments are needed to enhance our understanding of the role of *apo*-Fur regulation in virulence. Given that sRNAs have been shown in other systems to regulate genes once thought to be regulated directly by Fur, future studies are proposed to investigate the presence of *H. pylori* sRNAs involved in regulation of the predicted *apo*-Fur regulon. The proposed investigations could utilize previously characterized *H. pylori* Fur mutants or the heterologous constructs examined in Chapter 2 to examine the effects of *apo*-Fur on colonization and disease development. If this *Δapo*-Fur strain is shown to affect colonization or host pathology, direct examination of the 16 genes proposed to be in the *apo*-Fur regulon could provide insight into the development of therapeutic agents to prevent infection or combat disease. In addition, study of the interaction of *H. pylori* with the host immune response could also lead to the development of vaccines or other agents to prevent infection or disease. *H. pylori* has the distinction of persistently colonizing the harsh environment of the gastric mucosa in over half of the world's population. Despite the availability of treatment, once the infection is diagnosed, *H.*

pylori is still implicated in the development of gastric cancer in many individuals. In order to effectively prevent colonization and disease development, it is important to understand how this bacterium, in the face of a relative paucity of transcriptional regulators and two component systems, is able to adapt and survive the tumultuous gastric environment.

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