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MINIREVIEW

This Is Not Your Mother's Repressor: the Complex Role of Fur in Pathogenesis▽

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In the struggle between host and pathogen, competition for resources is often a key point in determining who will be the ultimate winner. The goal of the pathogen is to secure the necessary resources, often nutrients, from the host, while the goal of the host is to sequester the utilizable resources from the pathogen to help prevent infection. Among the key nutrients necessary to virtually all forms of life is iron. Iron plays an essential role in a diverse number of cellular processes. For instance, it serves as an enzymatic cofactor in metabolism and for electron transport. Thus, obtaining sufficient amounts of iron and maintaining iron stores are critical functions for both pathogen and host. However, having too much iron can be detrimental, as excess iron can lead to the formation of hydroxyl radicals through Fenton chemistry, which in turn may lead to cellular and DNA damage.

Based on this yin and yang relationship, it is perhaps no surprise that the human host has several mechanisms for sequestering iron. In the body, iron is stored primarily in ferritin and hemosiderin while the majority of functional iron is found in hemoglobin (4). This being said, there are several other iron storage molecules, like lactoferrin and transferrin, which sequester iron at the mucosal surfaces and within the circulatory system, respectively, and have been found to be iron sources for some pathogens (95). Global sequestration of free iron prevents possible oxidative damage as well as easy acquisition of free iron by pathogenic microbes. Additionally, in a further attempt to limit iron availability to pathogens during infection, the host decreases iron absorption from the gut, increases production of iron storage molecules, and shifts iron from the plasma into the storage molecules (123). Also, iron storage molecules are positioned in areas that are likely to be sites of infection. Thus, the host is immediately able to remove iron from those sites if a pathogen is detected (123). This process of removing free iron and other nutrients from the body and containing them in various storage molecules is termed "nutritional immunity" (124).

Despite these well-orchestrated defenses, bacterial pathogens have evolved mechanisms to breach the iron stores as well as to compete with the host for free iron. Proof of their success can be found by the strong connection between host iron overload and increased susceptibility to several bacterial infections. Indeed, in a recent review of 67 years of medical literature, Khan et al. found an increased association between infection with bacterial pathogens, such as *Escherichia coli*, *Listeria monocytogenes*, and *Vibrio vulnificus*, and hemochromatosis (84). In addition, iron overload in hemodialysis patients is associated with an increased number of bacterial infections as well as an increase in septicemic episodes (118). The importance of iron and infection in humans has also been validated for multiple pathogens by use of animal models. For instance, in a murine model of *V. vulnificus* infection, there is a drastic decrease in the 50% lethal dose, from 10^6 to 1.1 bacterial cells, in mice injected with extra iron (127). Also, *L. monocytogenes* exhibits increased growth in vivo and displays a decreased 50% lethal dose in mice given additional iron (116). Finally, when excess iron is introduced in murine infection models for both *Neisseria meningitidis* (76) and *Salmonella enterica* serovar Typhimurium (85), infection is enhanced. While this is by no means an exhaustive list, it is clear that excess iron in the host helps to create a more hospitable environment for opportunistic pathogens. This is likely due to an increase in available free iron and potentially a decrease in antibacterial leukocyte activities (123).

In the midst of the mounting evidence for the connection between iron availability and increased susceptibility to bacterial infection, a mutant of *S.* Typhimurium that showed constitutive high-level expression of the iron-enterochelin and ferrichrome iron uptake systems was isolated (46). This strain was named the *fur* mutant, for iron (*F*e) *u*ptake *r*egulation (46), and today represents what we know as the ferric uptake regulator. The first *fur* mutants of *E. coli* were identified in 1981 and showed constitutive expression of *cir*, *fhuA*, and *fecA*, three iron uptake systems that are typically upregulated when available iron is low (71). Within 1 year, *E. coli fur* complementation studies showed that *fur* carried on an F′ *lac* plasmid restored the wild-type phenotype in a strain bearing a chromosomal *fur* mutation (70). *E. coli fur* was successfully cloned in 1984 (69), and the gene sequence was derived shortly thereafter (112). Sequence and biochemical analyses went on to show that Fur is conserved across a wide range of bacterial species and is a small regulatory protein (15 to 17 kDa, approximately 150 amino acids) that functions as a dimer, is cofactored by Fe(II), and is usually autoregulatory.

A greater understanding of the mechanism of Fur regulation

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came with the first description of a DNA binding consensus sequence for *E. coli* Fur (39). This 19-bp consensus sequence, GATAATGATAATCATTATC (39), became the gold standard for comparison of types of Fur regulation across bacterial species and facilitated the understanding of exactly how Fur functions as a regulator. When iron is readily available in the bacterial cell, Fur binds iron and dimerizes, and the iron-bound Fur dimers bind to the consensus sequence in target promoters. Binding of Fur at the promoters prevents the binding of RNA polymerase; thus, transcription of the target gene is prevented. While Fur was first characterized as a transcriptional repressor under iron-abundant conditions, it has subsequently been shown to function as an activator and even to repress certain genes in the absence of the iron cofactor; these diverse types of Fur regulation are discussed in further detail below (see Fig. 1). While *fur* regulation continued to be studied for *E. coli* and for a wide variety of bacteria, it would take 25 years before what was arguably the next big breakthrough in the study of this regulator occurred—the crystallization of *Pseudomonas aeruginosa* Fur (106). Having the crystal structure of this important regulatory protein has enabled researchers to begin to make connections between what is known from genetic studies and the actual structure of the protein.

Fur, IRON HOMEOSTASIS, AND BACTERIAL SURVIVAL

Since iron is an essential nutrient for nearly all bacterial life but deadly in excess quantities, Fur's regulation of iron uptake and storage genes plays a significant role in the lives of the diverse number of bacteria that utilize it. As *E. coli* Fur is among the best studied, there have been numerous publications detailing Fur regulation of iron uptake systems in this model organism. These include the ferric citrate transport system (*fecABCDE*), the ferrichrome-iron receptor (*fhuA*), the colicin I receptor (*cir*) (63, 69, 71), the regulator of the *fecABCDE* operon (*fecIR*) (3), the ferrienterochelin receptor (*fepA*), the ferric ion uptake gene (*fiu*) (69), the aerobactin (*iucA*) operon (5, 39, 47, 50), and the divergent operons of the ferrienterochelin receptor-ferric enterobactin esterase (*fepA-fes*) (49, 79). Additionally, recent macroarray analyses of the iron-dependent and Fur-dependent regulons in *E. coli* have confirmed the previously characterized Fur regulatory targets as well as identified several new Fur targets (92).

The large number of iron uptake genes that have been found to be controlled by Fur regulation is not restricted to gram-negative bacteria. Indeed, iron uptake is also regulated by Fur in the model gram-positive organism *Bacillus subtilis*. The best characterized of these systems include the catecholate siderophore *dhb* and a gene involved in ferrihydroximate transport, *fhuD* (19). Interestingly, despite the fact that in vivo repression of *dhb* is seen only in the presence of iron, in vitro Fur binds to the *dhb* promoter even in the absence of its iron cofactor, though with slightly lower affinity than when iron is present (18). Like with *E. coli*, global microarray analysis identified as many as 20 Fur-regulated operons, the majority of which are involved in iron acquisition (7).

Countless studies have gone on to show that, like with *E. coli* and *B. subtilis*, Fur plays an essential role in iron acquisition systems and many other homeostatic processes for numerous bacterial pathogens. While these are too numerous to discuss exhaustively here, a few key examples that illustrate genes involved in siderophore production, iron acquisition from heme, and iron storage are detailed. A more extensive list of pathogens that utilize Fur and their Fur-regulated genes is summarized in Table 1. However, once again, due to space limitations and the large volume of research on this important regulator, this table is by no means an exhaustive list.

Siderophores are iron-binding proteins secreted by bacteria to acquire iron from the environment. In *P. aeruginosa*, Fur regulates the production of the siderophores pyoverdin and pyochelin (107). In fact, pyoverdin has been found in the sputum of cystic fibrosis patients infected with *P. aeruginosa* (64), and isolates from patients produce both siderophores (65). A siderophore transport system (*sir*) and the ferrichrome uptake operon (*fhu*) are also regulated by Fur in *Staphylococcus aureus* (78, 130). Next, Fur regulates genes involved in the acquisition of iron from unique host sources, like heme and transferrin. *V. vulnificus* Fur regulates *hupA*, a heme utilization gene (89), while the causative agent of human chancroid, *Haemophilus ducreyi*, utilizes Fur to control the expression of *hgbA*, which encodes a protein involved in hemoglobin binding (24). It is interesting to note that even though pathogenic *Neisseria* species produce transferrin receptors (*tbpA* and *tbpB*) to bind host iron sources, rather than producing siderophores to scavenge iron directly, the host may be attempting to limit iron availability and to decrease colonization by producing antibodies to these receptors during infection (1). Finally, Fur regulation of iron storage molecules is also important for bacterial pathogenesis to help ensure that once iron is acquired from the host, it is stored for use by the bacteria and contained to prevent the toxic effects of excess free iron. *fri*, the only identified ferritin-like protein in *L. monocytogenes*, is Fur regulated (52), as is a seven-gene locus, *svpA-srtB*, that is likely involved in iron uptake (97). Additionally, *Helicobacter pylori* Fur has been shown to regulate genes involved in iron acquisition and storage. The *pfr* gene, which encodes a prokaryotic ferritin molecule, is repressed by Fur in the absence of iron in what is termed *apo*-Fur regulation (14, 37), while iron uptake systems encoded by *frpB* (33, 37, 120), *fecA* (120), *ceuE* (120), *feoB* (120), and *exbB* (32) have all been found to be repressed by Fur in the presence of iron.

Not only does Fur play a role in iron acquisition in animal pathogens, but it has been found to be important for plant pathogens as well. In *Pseudomonas syringae*, Fur represses siderophore production (25), and in *Bradyrhizobium japonicum*, Fur regulates *irr*, the regulator of the heme biosynthetic pathway (56, 68). Even from this limited list of pathogens, it is clear that Fur is critical for iron acquisition and storage in a wide variety of bacterial species.

Fur AND THE LINK TO VIRULENCE

Not only is Fur involved in regulating iron homeostasis, it is also more directly involved in colonization and virulence. *fur* mutants of *H. pylori* are less efficient at colonization than the wild type in a murine model of infection (20) and are easily outcompeted by wild-type bacteria in in vivo competition assays with a Mongolian gerbil model of infection (58). Thus, while it is not an essential gene in *H. pylori*, Fur certainly provides an advantage in establishing colonization. In addition,

TABLE 1. Diverse Fur-regulated genes from model organisms and bacterial pathogens[a]

Organism	Gene name(s) and description	Type of Fur regulation	Reference(s)
B. subtilis	*dhb*, catecholate siderophore	Fe-Fur repression	18, 19
	fhuD, ferri-hydroximate transport	Fe-Fur repression	19
B. japonicum	*irr*, heme biosynthetic pathway regulator	Fe-Fur repression	56, 68
C. jejuni	Cj0859c, hypothetical	Suspected *apo*-Fur repression	77
	Cj1364, fumarate hydratase	Suspected *apo*-Fur repression	77
E. coli	*bfr*, bacterioferritin	Indirect Fur activation	91
	cfaB, CFA/I fimbrial subunit gene	Fe-Fur repression	82
	cir, colicin I receptor	Fe-Fur repression	63, 69, 71
	fecABCDE, ferric citrate transport	Fe-Fur repression	3, 69, 71
	fecIR, regulator of *fecABCDE* operon	Fe-Fur repression	3
	fepA, ferrienterochelin receptor	Fe-Fur repression	49, 69, 79
	fes, ferric enterobactin esterase	Fe-Fur repression	49, 79
	fhuA, ferrichrome-iron receptor	Fe-Fur repression	69, 71
	fiu, ferric ion uptake	Fe-Fur repression	69
	fur, ferric uptake regulator	Fe-Fur repression	38
	hly, hemolysin	Fe-Fur repression	55
	iha, IrgA homolog adhesin	Fe-Fur repression	110
	iucA, aerobactin	Fe-Fur repression	5, 39, 47, 50
	sltA and *sltB*, Shiga toxins (Shiga-like toxins)	Fe-Fur repression	21
	sodA, Mn-containing superoxide dismutase	Fe-Fur repression	11, 27, 72, 98, 108, 117
	sodB, Fe-containing superoxide dismutase	Indirect Fur activation	41, 42, 98
H. ducreyi	*hgbA*, hemoglobin binding protein	Fe-Fur repression	24
H. pylori	*amiE*, aliphatic amidase	Fe-Fur repression	23, 119
	ceuE, periplasmic iron binding protein	Fe-Fur repression	120
	exbB, biopolymer transport protein	Fe-Fur repression	32
	fecA, ferric citrate transport	Fe-Fur repression	120
	feoB, ferrous iron transport	Fe-Fur repression	120
	frpB, iron uptake system	Fe-Fur repression	33, 37, 120
	fur, ferric uptake regulator	Fe-Fur repression, *apo*-Fur activation	35, 36
	nifS, Fe-S cluster synthesis protein	Fe-Fur activation	2
	pfr, prokaryotic ferritin	*apo*-Fur repression	14, 23, 37
	sodB, Fe-containing superoxide dismutase	*apo*-Fur repression	23a, 45
	vacA, vacuolating cytotoxin	Indirect Fur repression	58
L. monocytogenes	*fri*, ferritin-like protein	Fe-Fur repression	52
	fur, ferric uptake regulator	Fur regulation	86
	svpA-srtB, iron uptake locus	Fe-Fur repression	97
M. smegmatis	*kat*, catalase-peroxidase	Fe-Fur repression	109, 132
M. tuberculosis	*kat*, catalase-peroxidase	Fe-Fur repression	109, 132
N. gonorrhoeae	*fbpA*, periplasmic binding protein	Fe-Fur repression	40
	fur, ferric uptake regulator	Fe-Fur repression	113
	opaA, *opaB*, *opaC*, *opaD*, *opaF*, *opaG*, *opaJ*, *opaK*, *opaE*, *opaH*, and *opaI*, opacity proteins	Fe-Fur repression	113
	sodB, superoxide dismutase	Fur activation	113
	tbpA and *tbpB*, transferrin receptors	Fe-Fur repression	1
	tonB, receptor	Fe-Fur repression	113
N. meningitidis	*norB*, nitric oxide reductase	Fe-Fur activation	34
	nuoA, NADH dehydrogenase I chain A	Fe-Fur activation	34
	pan1 (now referred to as *aniA*), anaerobically induced outer membrane protein	Fe-Fur activation	34
P. aeruginosa	*bfr*, bacterioferritin	Fe-Fur activation	126
	fhuA, ferrichrome-iron receptor	Fe-Fur repression	99
	katB, catalase	Indirect Fur repression	74
	pchR, pyochelin siderophore	Fe-Fur repression	99

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TABLE 1—*Continued*

Organism	Gene name(s) and description	Type of Fur regulation	Reference(s)
	pfeR, enterobactin receptor regulator	Fe-Fur repression	100
	pvdS, alternate sigma factor	Fe-Fur repression	99
	sdh, succinate dehydrogenase	Indirect Fur activation	126
	sodA, Mn-containing superoxide dismutase	Fe-Fur repression	73, 74
	sodB, Fe-containing superoxide dismutase	Indirect Fur activation	126
	tonB, receptor	Fe-Fur repression	100
	toxA, exotoxin A	Indirect Fur repression	99, 107
S. aureus	*fhuCBD*, ferrichrome-iron receptor	Fe-Fur repression	78, 130
	kat, catalase-peroxidase	Fur activation[b]	78
	sirABC, siderophore transport system	Fe-Fur repression	78, 130
S. coelicolor	*catC*, catalase-peroxidase	Fe-Fur repression	66
	fur, ferric uptake regulator	Fe-Fur repression	66
S. enterica serovar Typhimurium	*hmp*, flavohemoglobin	Fe-Fur repression	29
	iro-28, iron-regulated protein	*apo*-Fur activation	54, 67
	mntH, bacterial homolog of mammalian natural-resistance-associated macrophage protein 1	Fe-Fur repression	80, 83
	rfrA and *rfrB*, sRNA	Fe-Fur repression	43
	sodB, Fe-containing superoxide dismutase	Indirect Fur activation	43
V. cholerae	*hly*, hemolysin	Fe-Fur repression	114
	irgA, outer membrane protein	Fe-Fur repression	60, 90
V. vulnificus	*fur*, ferric uptake regulator	*apo*-Fur activation	87
	hupA, heme utilization gene	Fe-Fur repression	89
	vuuA, vulnibactin receptor	Fe-Fur repression	122
Y. pestis	*bfr*, bacterioferritin	Fe-Fur repression	59
	fhuCDB, ferrichrome-iron receptor	Fe-Fur repression	59
	feoAB, ferrous iron transport	Fe-Fur repression	59
	fepB, ferrienterochelin receptor	Fe-Fur repression	59
	ftnA, iron storage protein	Fe-Fur activation	59
	iucA, aerobactin biosynthesis protein	Fe-Fur repression	59
	katA, catalase	*apo*-Fur activation	59
	napF, ferredoxin-type protein	Fe-Fur activation	59
	tonB, receptor	Fe-Fur repression	59

[a] Due to the large volume of research on Fur, this table does not represent an exhaustive list of Fur-regulated genes.
[b] Not determined whether Fur activation is mediated through Fe-Fur or *apo*-Fur.

fur mutants of several pathogens exhibit decreased virulence in animal models. A murine skin abscess model of *S. aureus* infection shows that *fur* mutants are attenuated (78). Likewise, *fur* mutants of *L. monocytogenes* (111) and *Campylobacter jejuni* (103) show reduced virulence in murine and chick models of infection, respectively, as do *Edwardsiella tarda fur* mutants in fish (121). Even in plant pathogens, like *P. syringae*, Fur mutants show decreased virulence (25).

In addition to playing a role in colonization, Fur regulates numerous genes that are important for bacterial pathogenesis. For instance, in *P. aeruginosa*, Fur has been shown to be involved in toxin production, biofilm formation, and quorum sensing. Fur is believed to be indirectly involved in *toxA* expression, since it does not interact with the promoter of either *toxA* or its regulator, *regAB* (99, 107). Although the role Fur plays in biofilm formation in *P. aeruginosa* is not well characterized, it has been shown that a *fur* mutant forms more-mature biofilms than does the wild type under iron-limited conditions (8). Finally, Fur is indirectly involved in the regulation of quorum sensing in *P. aeruginosa* through the regulation of two small, noncoding RNAs (sRNAs), *prrF1* and *prrF2*, which in turn regulate degradation enzymes for the precursor molecule to the *Pseudomonas* quinolone signal (101).

In *Vibrio cholerae*, Fur negatively regulates hemolysin production (114) and an outer membrane virulence determinant, *irgA* (60, 90), while in *Neisseria gonorrhoeae*, Fur has been shown to interact directly with the promoters of all 11 *opa* genes, which encode outer membrane proteins that aid in adherence to and invasion of host cells (113). In *N. meningitidis*, Fur is implicated in the regulation of several genes associated with virulence (62), and heat shock proteins are deregulated in a *fur* mutant of *N. meningitidis* independently of the iron-Fur regulon (31). In *E. coli*, the Shiga toxins (Shiga-like toxins) SltA and SltB (21) and hemolysin (55) are repressed by Fur. Additionally, in uropathogenic and enterohemorrhagic strains of *E. coli*, Fur negatively regulates the IrgA homolog adhesin (*iha*) (110), while in enterotoxigenic *E. coli*, expression of the fimbrial adhesin CFA/I is repressed by Fur (82). Finally, the vacuolating cytotoxin (*vacA*) of *H. pylori* has been shown to be indirectly regulated by Fur (58). Thus, Fur plays a role in colonization and virulence in a diverse number of pathogens.

Fur AND LOW PH

In addition to its role in the regulation of virulence factors, Fur is important for the regulation of processes that are necessary for survival in vivo and thus are linked to virulence. For instance, Fur is an important regulator of genes involved in the acid resistance response. Arguably, this is best exemplified in *S.* Typhimurium, where Fur is involved in the acid tolerance response (ATR). Specifically, an *S.* Typhimurium *fur* mutant is unable to mount an effective ATR at pH 5.8 (53). Therefore, *fur* mutants are more sensitive to acid (pH 3.3) than the wild type, and several ATR genes are not induced at pH 5.8 in the absence of *fur* (53). Intriguingly, Fur's role in acid resistance appears to be independent of iron and its role in iron acquisition (67), as iron availability does not affect the ATR and "iron-blind" *fur* mutants still display an acid resistance phenotype (67). Further work has shown that Fur is involved primarily in helping *S.* Typhimurium combat organic acid stress but plays only a minor role in inorganic acid stress (10). Fur in *H. pylori* has also been implicated in regulating genes involved in fighting acid stress (17, 58, 93); in fact, when the organism is exposed to low pH, the number of genes in the Fur regulon is significantly increased (58). These genes include *gluP*, encoding a predicted glucose/galactose transporter; *ruvC*, encoding a predicted Holliday junction endodeoxyribonuclease; *fliP*, encoding a flagellar biosynthetic protein; and *amiE*, encoding the aliphatic amidase (58). AmiE helps counteract acid stress through the production of ammonia as a by-product of the hydrolysis of aliphatic amides (119). While Fur in these organisms is not solely responsible for acid resistance, it certainly plays a significant role in helping the bacteria adapt and adjust to acidic conditions that would be encountered within the host.

Fur AND OXIDATIVE STRESS

Another survival mechanism in which Fur has been found to play a role in pathogenesis is fighting oxidative stress via regulation of genes, like catalase and superoxide dismutase, that help to combat toxic oxygen products. Catalases and hydroperoxidases convert peroxides into water and oxygen, and superoxide dismutases convert superoxide radicals into oxygen and peroxide. For instance, in several organisms, the catalase (*kat*) gene, which encodes the catalase enzyme, is regulated by Fur. Fur represses *katG*, a combined catalase-peroxidase, in both *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*, and this regulation is predicted to be universal in all *Mycobacterium* species (109, 132). Some bacterial species, like *Yersinia pestis* (59), *S. aureus* (78), and *P. aeruginosa* (74), also utilize Fur to activate *kat* expression. *E. coli fur* mutants are more susceptible to UVA irradiation oxidative damage due to decreased production of the hydroperoxidases (HPI and HPII) (75). The neutrophil activating protein (*napA*), which helps to protect *H. pylori* from oxidative damage, is suspected to be under the control of Fur (28, 94, 102). In addition, *S.* Typhimurium Fur helps the bacterium counteract the effects of nitric oxide stress through the regulation of *hmp*, a flavohemoglobin (29). Also of interest for this organism is the Fur regulation of *mntH*, a gene that encodes a bacterial homolog of mammalian natural-resistance-associated macrophage protein 1 (80, 83). MntH is thought to help *S.* Typhimurium fight hydrogen peroxide-related injury upon entrance into macrophages (83).

Another oxidative survival gene that is commonly regulated by Fur is superoxide dismutase (*sod*). Superoxide dismutases are classified based on their metal cofactor: SodA, SodB, and SodC utilize Mn(II), Fe(II), and Cu(II) or Zn(II), respectively. The type of Sod varies with the bacterial species. In *N. gonorrhoeae*, Fur directly binds to the *sod* promoter, which results in *sod* activation (113), while in *E. coli*, *sodA* is directly repressed by Fur under iron-abundant conditions (11, 27, 72, 98, 108, 117). In comparison, the iron superoxide dismutase (*sodB*) in *E. coli* is indirectly activated by Fur (41, 42, 98). As is seen here, methods of Fur regulation of *sod* genes are exceptionally diverse, and to add to this mixture of direct or indirect activation and direct repression, there is one more manner in which Fur has been shown to regulate *sod*. In *H. pylori*, *sodB* is directly repressed by Fur in the absence of iron, i.e., in its *apo* form (45).

It is evident that Fur is a global regulator that is involved in bacterial pathogenesis as well as in many aspects of bacterial life (even some not described here, e.g., Fur regulation of metabolic genes). As mentioned, classical Fur regulation involves the binding of iron-bound Fur dimers to the promoter region of target genes to occlude the RNA polymerase binding site; however, as also mentioned above, recent studies have shown instances where Fur functions as an activator or as a repressor in the absence of its iron cofactor. Presently, only one organism utilizes Fur in all of these different ways—*H. pylori*. While iron-bound Fur repression in this organism is well understood, *apo*-Fur repression and Fur activation comparatively remain in the proverbial "black box." In the remainder of this review, we highlight and compare the complexities of Fur regulation in this important human pathogen.

H. PYLORI AND IRON-ASSOCIATED DISEASE

Interestingly, *H. pylori* infections are often associated with development of an iron deficiency anemia (IDA) that is usually unresponsive to iron replacement therapies (9). Two recent epidemiological studies highlighted this link by looking at adolescents and pregnant women, two groups of people who are at increased risk for IDA. During the adolescent years, an increased amount of iron is needed to support the rapid growth of the child, and similarly, during pregnancy, women need more iron due to increased blood volume and the iron needs of the developing fetus. In the first study, three adolescent children with IDA were unresponsive to iron supplementation (22). After the teens were found to be infected with *H. pylori* and the infections were eradicated, the anemia resolved and iron levels returned to normal by 3 months posttreatment (22). In the second study, a link between IDA in pregnancy and *H. pylori* infection was made. Out of 117 pregnant women, 27 had anemia; 18 were classified as suffering from IDA (96). All 27 of the anemic patients were shown to be *H. pylori* infected (96). The close association between *H. pylori* infection and IDA prompted Cardamone et al. to suggest that in cases of refractory IDA in teens, *H. pylori* infection should be considered as a diagnosis even in the absence of gastric symptoms (22).

While there is a strong epidemiological association between *H. pylori* and IDA, the mechanism by which the bacterium

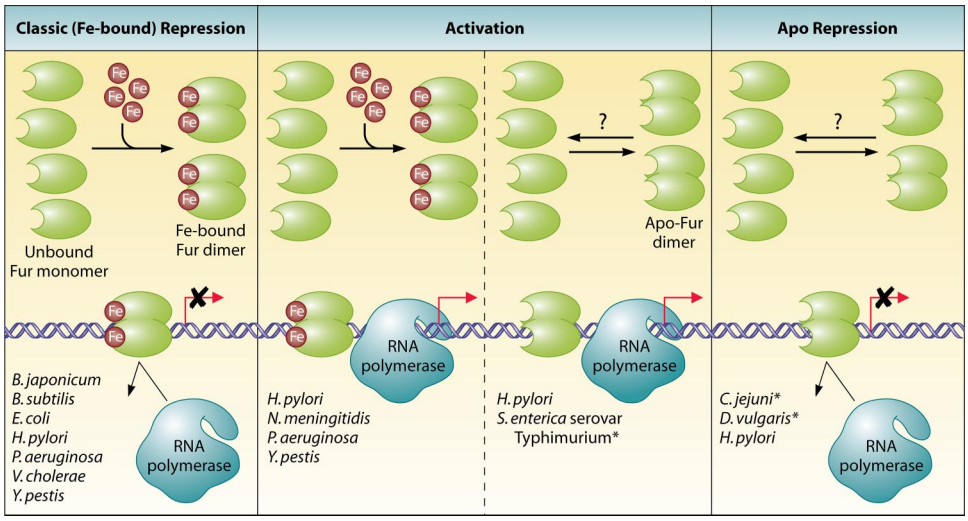

FIG. 1. Basic features of Fe-Fur repression, *apo*-Fur repression, and Fur activation. Characteristic features of each type of Fur regulation are shown as they interact with a target DNA promoter. (Left) Classical iron-bound Fur repression. As iron becomes increasingly available in the bacterial cell, the Fe(II) cofactor binds to *apo*-Fur monomers, and these now-iron-bound monomers dimerize. The iron-bound Fur dimers repress transcription by binding to the Fur box in their target promoters and block the binding of RNA polymerase. (Center) Iron-bound Fur and *apo*-Fur activation. On the left, iron-bound Fur dimers are formed under conditions of iron abundance, and these dimers bind to Fur boxes in their respective target promoters and activate gene transcription. On the right, *apo*-Fur dimers form under low-iron conditions. These *apo*-Fur dimers bind to Fur boxes in their target promoters and activate gene transcription. (Right) *apo*-Fur repression. Under iron depletion conditions, Fur is in its *apo* form, and *apo*-Fur binds to the Fur boxes of its target promoters. This binding blocks the binding of RNA polymerase; hence, transcription is repressed. For the sake of simplicity, *apo*-Fur repression and activation are depicted as being mediated through an *apo*-Fur dimer, although it is not known whether *apo*-Fur functions as a monomer or a dimer. Abbreviated lists of organisms that utilize each type of Fur regulation are listed in each panel. An asterisk indicates organisms for which *apo*-Fur regulation has been suggested but direct interaction has not yet been determined.

causes IDA is not known—does the bacterium directly remove iron from the host, or is the severe inflammation associated with the infection the source of the iron loss? Several studies of *H. pylori* strains isolated from patients with IDA have attempted to explain the epidemiological association. Proteomic analysis of 15 strains (7 from IDA patients and 8 from non-IDA patients) revealed that IDA strains phylogenetically clustered together and separately from the non-IDA strains (105). Additionally, in a study of IDA strain isolates compared to non-IDA isolates, the strains from IDA patients showed increased uptake of both Fe(II) and Fe(III) (131); while the reason for this increased iron uptake is not known, certain polymorphisms in *feoB*, a ferrous iron transporter, have been shown to occur in IDA-derived strains of *H. pylori* (81). Even though the exact mechanism by which *H. pylori* and IDA are linked is not well understood, it is highly likely that Fur plays some role in this process, as it is the primary regulator of iron uptake and storage genes in this organism.

IRON-BOUND Fur REPRESSION

Fur was first identified in *H. pylori* in 1998 (16), is conserved (over 95% identical at the DNA and amino acid levels) among *H. pylori* strains (15), and is 34% identical and 56% similar to *E. coli* Fur. Although Fur is nonessential in *H. pylori* (26), as discussed above, it has been shown to be important for efficient colonization (20, 58). Based on the strong similarity between *H. pylori* Fur and *E. coli* Fur, it is likely no surprise that aspects of Fur regulation are similar for *H. pylori* and *E. coli*. As mentioned above, the best-described means of Fur regulation is classically hallmarked by iron-bound Fur dimers binding to specific regions in iron-regulated promoters called Fur boxes (Fig. 1, left). Fur binding blocks the binding of RNA polymerase, thus preventing transcription of these target genes (47, 49). In *E. coli*, the Fur box is a 19-bp region, GATAATGAT AATCATTATC, that is highly conserved in this organism (39). The *E. coli* Fur box has also been reevaluated as three repeats of GATAAT, with the second and third repeats separated by a single nucleotide and the last repeat inverted (48). Although the Fur box of *E. coli* is used as the standard to which other Fur binding sequences are compared, it is not clearly conserved in all organisms that exhibit Fur regulation. For example, in *B. subtilis*, the Fur box is a 15-bp inverted repeat in a 7-1-7 configuration (6). Two of these motifs ($[7\text{-}1\text{-}7]_2$) may overlap to form the classic 19-bp *E. coli* sequence (6). In *Y. pestis*, the Fur box consists of two inverted repeats of AATGATAAT separated by a single nucleotide (59). One common feature among Fur boxes is the high number of A/T nucleotides relative to C/G nucleotides. Thus, for *H. pylori* it is perhaps no surprise that the definition of a consensus Fur box is somewhat hindered by the fact that it is a highly A/T-rich organism (approximately 60%). Based on an alignment of several Fur-regulated genes, the consensus Fur box in *H. pylori* is NNNNNAATAA TNNTNANN (94). This consensus sequence is significantly different from that for *E. coli* and is certainly less conserved, even among *H. pylori* Fur-regulated genes, than the *E. coli* sequence. While it is currently unclear, it may be that the requirement for Fur binding is less reliant on a recognition sequence and more related to the overall structural configuration of the target promoter sequence in *H. pylori.* This notion

is further supported by the fact that *H. pylori* Fur is only partially able to complement an *E. coli fur* mutant (14) and that an *E. coli* Fur titration assay (FURTA-Ec) was not very successful at identifying Fur-regulated genes in *H. pylori* (12, 15, 16) until the system was modified to allow *H. pylori* Fur expression (51).

Even though all of the specifics are not known, iron-bound Fur repression in *H. pylori* has been well documented, and binding to several gene targets has been confirmed through DNase footprinting analysis. Indeed, the predicted Fur regulon in *H. pylori* is quite extensive (30, 44, 94). The regulon includes iron uptake genes, like *frpB* and *exbB* (32, 33, 37) among others, and *amiE*, as well as other genes involved in functions like acid resistance (23, 119). Generally, iron-bound Fur-regulated genes in *H. pylori* have one to three Fur binding sites within their promoters (32, 33, 37). The sites with the highest affinity span the −10 and/or −35 promoter element; the lower-affinity Fur binding sites are located further upstream from the primary Fur box (32, 33, 37). This high-affinity orientation supports the current hypothesis of Fur competing with RNA polymerase for binding to target promoters. Indeed, what we know about Fe-bound Fur regulation in *H. pylori* agrees with what is seen for many other organisms and is the most common mechanism of Fur regulation.

apo-Fur REGULATION

Currently unique to *H. pylori* is the utilization of Fur as a repressor even in the absence of its Fe(II) cofactor. This phenomenon is termed *apo*-Fur regulation. It occurs under conditions of low iron availability and involves iron-free Fur binding to target promoters to prevent the binding of RNA polymerase. The *apo*-Fur regulon consists of an entirely different set of genes than the Fe-bound Fur regulon and is predicted to contain approximately 16 genes (44), though few genes have definitively been shown to be regulated in this manner. Expression of the iron storage molecule Pfr is regulated by *apo*-Fur (14, 23, 37); *pfr* expression is repressed under conditions of low iron but is constitutively expressed in a *fur* mutant (14). DNase I footprinting analysis of the *pfr* promoter using iron-free Fur revealed that there were three regions of protection (37). As with iron-bound Fur repression, the region with the highest affinity for Fur covered the region to which RNA polymerase would bind (in this case, both the −10 and −35 promoter elements) (Fig. 1, right) (37). The other two regions were further upstream from the transcriptional start site (37). From a bacterial standpoint, repression of *pfr* under iron-limited conditions makes biological sense, as producing a storage molecule when the molecule to be stored is not available would be a waste of energy and resources.

Another confirmed *apo*-Fur target is *sodB.* Binding of Fur to *sodB* in the absence of iron was shown via DNase I footprinting analysis and electrophoretic mobility shift assays (EMSAs) (45). Unlike *pfr*, the *sodB* promoter has only one Fur binding region, which spans the −10 and −35 promoter elements (45). Interestingly, comparison of the three *pfr* Fur boxes and the *sodB* Fur box shows very little sequence homology between them. Additionally, there is little homology with the known iron-bound *H. pylori* Fur boxes and even less homology with the *E. coli* consensus Fur binding sequence (37, 45). Recent work from our group suggests that there are strain-specific nucleotide differences in the recognition sites in *apo*-Fur-regulated promoters and that these differences may alter the affinity of *apo*-Fur for these promoters; a single nucleotide difference in the *sodB* Fur box in strain G27 results in the loss of *sodB* regulation (23a).

Even with the direct binding data provided by DNase I footprinting and EMSA of the *pfr* and *sodB* promoters, the concept of *apo*-Fur regulation remains widely debated in the Fur field. The debate centers around whether or not Fur could actually be found unbound to iron in vitro. Is it possible to strip Fur of all of its iron cofactor in the laboratory? Some argue that the DNase footprinting data are artificial because it is impossible to create *apo*-Fur in vitro; however, it is clear from mutational and transcriptional analyses that genes in the "*apo*-Fur" regulon are repressed in the absence of iron and constitutively expressed in a *fur* mutant, regardless of iron availability. One possibility is that the existence of Fur-regulated sRNAs, which control *apo* targets, could explain the in vivo data. sRNAs are a subclass of natural antisense transcripts that base pair with complementary mRNA transcripts and thus can alter the stability of the mRNA or its ability to be translated (115). Up until very recently, there were no identified sRNAs in *H. pylori*, but recently four have been identified (128, 129). Two of the natural antisense transcripts in *H. pylori*, NAT-39 and NAT-67, were found to be complementary to *frpB* and *ceuE*, respectively (129). Both of these genes are members of the iron-bound Fur regulon (94). While it has been shown that NAT-39 and NAT-67 bind to their respective targets, it has not yet been determined what regulatory role this plays in gene expression and in iron homeostasis (129). The only other sRNAs identified in *H. pylori*, IG-443 and IG-524, are predicted to regulate the flagellar motor switch gene (*fliM*) and fumarase (*fumC*), respectively (128). Interestingly, IG-443 is encoded in the intergenic region between *fur* and HP1033 (128). Given that the existence of sRNAs in *H. pylori* is a very recent discovery, the possibility of an sRNA that could regulate genes in the *apo*-Fur regulon cannot be ruled out; however, to date there is no strong evidence of this being the case. Regardless, regulatory sRNA cannot account for the direct in vitro binding data demonstrated for *apo*-Fur and the *pfr* and *sodB* promoters.

Interestingly, there is some evidence that *apo*-Fur regulation may be found in other bacterial species. Microarray analysis of *C. jejuni* revealed that Cj1364, fumarate hydratase, and Cj0859c, a hypothetical protein, had reduced expression under iron-replete conditions and had increased expression in the *fur* mutant (77). More recently, microarray analysis of the nonpathogen *Desulfovibrio vulgaris* Hildenborough predicted that there are nine genes which are repressed by iron-free Fur (13). More-specific studies need to be performed to determine whether *apo*-Fur regulation actually occurs in these organisms.

AUTOREGULATION OF Fur

While some organisms have additional regulatory proteins to regulate Fur expression, like the catabolite activator protein in *E. coli* (38), RpoS in *V. vulnificus* (88), and NikR in *H. pylori* (32), autoregulation of Fur is the most conserved mechanism of *fur* regulation. Fur represses its own expression under iron-

replete conditions. Biologically speaking, it makes sense to link the expression of Fur to the level of available iron, given the dangers of iron toxicity. Fur can be thought of as a rheostat that senses the available iron and responds by regulating its own expression accordingly (35, 36). It was determined early on that *E. coli* Fur was autoregulatory (38). Similarly, Fur from *E. tarda* (121) and Fur from *N. gonorrhoeae* (113) are autoregulatory, and Fur from *Streptomyces coelicolor* is predicted to be autoregulatory (66). In all of these instances, Fur autoregulation is the straightforward classical iron-bound Fur repression. However, in some organisms, Fur autoregulation appears to be more complex. For example, for *L. monocytogenes*, Northern blot analysis reveals that *fur* is upregulated under iron-limited conditions, yet in vitro DNase I footprinting analysis shows that Fur is able to bind to and protect the Fur box region of the *fur* promoter in the absence of the metal cofactor (86). The authors suggest that these results indicate that Fur binding is also dependent on an as-yet-unidentified factor (86). In contrast to this and iron-bound Fur autoregulation, *V. vulnificus* Fur has been shown to bind to and activate *fur* expression in the absence of iron (87).

Fur autoregulation in *H. pylori* may very well be the most complex Fur autoregulatory circuit known to date, since it combines both the classical iron-bound Fur repression and the *apo*-Fur activation that is exhibited in *V. vulnificus*. Initial studies by Delany et al. revealed that there were three Fur binding regions in the *H. pylori fur* promoter. In order from highest to lowest affinity for Fur, operator I spans nucleotides −34 to −66, operator II spans nucleotides +19 to −13, and operator III spans nucleotides −87 to −104 (35). The first two operators are likely to be involved in repression of the *fur* promoter, as they encompass both the −10 and −35 promoter elements, but the role of the third and farthest-upstream operator was initially unclear (35). In their subsequent work, Delany et al. showed that the third operator region was indeed important for Fur autoregulation and that it functions as a site for *apo*-Fur activation (similarly to the type for *V. vulnificus*). Additionally, operator I is involved in both iron-bound Fur repression and *apo*-Fur activation of expression through binding Fur in its respective forms (36). Which form binds is driven by the prevalence of iron, as both forms bind to this operator with equal affinities. The current model of *H. pylori* Fur autoregulation also suggests that if the concentration of Fur dips below a certain level, then Fur binding to operator I is lost, allowing this site to act as an UP element for RNA polymerase (36). Given that this organism utilizes Fur in both its iron-bound and *apo* forms, it is perhaps not surprising that Fur autoregulation in *H. pylori* is a complex mixture of iron-bound Fur repression and *apo*-Fur activation. Additionally, with few regulatory proteins relative to its genome size, *H. pylori* would likely have evolved to utilize every regulatory mechanism it has to ensure proper homeostasis.

Fur ACTIVATION

The complexity of *fur* autoregulation in *H. pylori* points to yet another regulatory function of Fur; Fur can act as a positive regulator. The first indication that Fur may act as a positive regulator came from microarray analyses where a number of genes were suggested to be Fur induced (30, 44). Another gene, *oorD*, a ferredoxin-like protein, is suspected of being activated by Fe-Fur, as its expression is decreased in the absence of iron, and EMSA shows that Fe-bound Fur binds to its promoter (58).

Despite this circumstantial evidence, the process of Fur activation in *H. pylori* is currently poorly understood, except with *nifS*. NifS is a Fe-S cluster synthesis protein that has been shown to be activated by iron-bound Fur (2). EMSA analysis shows Fur binding to the *nifS* promoter in the presence of the Fe(II) substitute Mn, and *nifS* expression is increased in the presence of iron (2). Interestingly, the two predicted Fur boxes for *nifS* are located far upstream of the transcriptional start site in the *nifS* promoter (2), similarly to the *apo*-Fur activation site within the *fur* promoter. It appears from the examples of *fur*, *nifS*, and possibly *oorD* that both iron-bound Fur and *apo*-Fur can act as transcriptional activators in addition to acting as repressors.

While there is clearly much to be learned about Fur activation in *H. pylori*, Fur activation in other organisms is better understood. For *N. meningitidis*, microarray analysis suggested that Fur activates multiple genes in the presence of iron (31, 62). Moreover, direct iron-bound activation of the NMB1436-NMB1438 operon (61, 62) and the *aniA*, *norB*, and *nuoA* promoters (34) has been shown. As with *H. pylori*, the Fur boxes for the Fur-activated genes in *N. meningitidis* are located further upstream in the promoters (34, 61). *S.* Typhimurium utilizes both iron-bound Fur and *apo*-Fur to activate a subset of genes, although whether this is direct or indirect activation remains unclear (54). Additionally, the iron-regulated protein IRO-28 appears to be activated by *apo*-Fur under iron-limited conditions in *S.* Typhimurium (54, 67). In *P. aeruginosa*, direct iron-bound Fur activation has been identified for the bacterioferritin gene *bfrB* (126).

It is clear from these examples that Fur activation does not occur in the same manner as Fur repression (Fig. 1, center). For both iron-bound Fur and *apo*-Fur repression, the Fur boxes are located near the transcriptional start site and usually span at least one of the key promoter elements. Binding at this location blocks the binding of RNA polymerase. In contrast, the Fur boxes for Fur-activated genes are all located far upstream from the transcriptional start site; thus, binding of RNA polymerase is not hindered. Perhaps by binding further upstream within the promoter, Fur is able to change the overall structure of the DNA, enabling better binding of the RNA polymerase to help facilitate transcription.

CONCLUSIONS AND FUTURE DIRECTIONS

More than 30 years since the first *fur* mutant was identified, Fur has gone from being thought of as a simple repressor of iron uptake genes to being considered a global regulator with multiple functions. This protein is still the source of much research and interest within the scientific community, which has gone beyond the classic field of regulation. For instance, with the increase in the field of bioinformatics Fur has been used as a model system to look at the identification of new targets (104). In addition, Fur has even been used to help identify whether fleas are infected with *Y. pestis* (57), indicating that this important regulatory protein could be used as a genetic marker in the diagnosis of other bacterial infections.

Finally, because it is an important global regulator, Fur could even be the future target of antibiotics or other antibacterial treatments (125).

As discussed here, Fur can act as either a repressor or an activator and function with or without its iron cofactor. For *H. pylori*, we find all of these different modes of Fur utilized within a single species. Based on this, it is perhaps not surprising that we also see that the most debate about Fur regulation stems from this organism. Although *apo*-Fur has been demonstrated to bind directly to target promoters in vitro, and in vivo transcriptional data indicate repression only in the absence of iron, as demonstrated by several groups (14, 23, 37, 45), to this day, *apo*-Fur repression remains a highly debated mechanism of action, and the possibility of *apo*-Fur actually existing in vitro remains a point of contention.

All debate aside, there are still several unanswered questions about Fur regulation in *H. pylori*. For instance, what does Fur recognize in its target promoters? There is no strong Fur box consensus sequence in this organism for any type of Fur regulation (iron-bound repression, *apo* repression, or activation). This may indicate that, in *H. pylori*, Fur recognizes more than just a specific DNA sequence. Perhaps there is something important about the overall three-dimensional structure of the DNA. This may be true of other organisms as well; *B. japonicum* Fur represses *irr*, and there is no clear Fur box within the *irr* promoter (56). The challenge to answering the question of what Fur recognizes in *H. pylori* promoters, at least for iron-bound Fur and *apo*-Fur repression, is that these Fur boxes commonly overlap/encompass the −10 and −35 promoter elements, making promoter mutation analysis virtually impossible.

For *H. pylori*, very little is understood about Fur activation and the mechanism of action for Fur with the activated promoter. While microarrays are able to globally analyze the Fur regulon, they are not capable of determining the specifics of Fur regulation; therefore, it is not clear from these types of studies whether Fur directly activates the identified genes or whether Fur indirectly regulates these targets. Further studies using EMSAs and/or DNase I footprinting need to be conducted on potential Fur targets to better determine the role of Fur in their activation. Also, how does Fur binding contribute to upregulating transcription? Does the binding of Fur alter the structure of the DNA to allow for the binding of RNA polymerase, or does Fur binding alter the binding site of a negative regulator of the target gene, thereby preventing the repressor from binding?

While the mechanism of iron-bound Fur regulation is well understood, comparatively little is known about *apo*-Fur regulation. For instance, it is not known whether *apo*-Fur functions as a monomer or a dimer. If it functions as a dimer, how does it compare to the structure of the iron-bound dimer? Another interesting question is what *apo*-Fur recognizes in its target promoters, since the current data do not suggest a conserved binding sequence. These questions, as well as the continued determination of the biological existence of *apo*-Fur, warrant further exploration. It will also be interesting to learn whether other species have *apo*-Fur repression or whether this type of regulation is specific to the evolution of *H. pylori*. Are there amino acid residues that are particularly important for one type of regulation or another? Structure-function analysis has not really been applied to Fur of *H. pylori*, and yet this organism utilizes Fur as a repressor and an activator in both its iron-bound and *apo* forms, which is not known to occur in any other bacterial species. Because of the diverse manners in which Fur impacts pathogenesis (through regulation of iron uptake systems, virulence factors, and oxidative and pH-mediated stress responses, etc.), understanding Fur regulation in *H. pylori* will enrich and broaden our understanding of the role Fur plays in the larger realm of bacterial pathogenesis.

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