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Functional specialization within the Fur family of metalloregulators

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Abstract The ferric uptake regulator (Fur) protein, as originally described in *Escherichia coli*, is an iron-sensing repressor that controls the expression of genes for siderophore biosynthesis and iron transport. Although Fur is commonly thought of as a metal-dependent repressor, Fur also activates the expression of many genes by either indirect or direct mechanisms. In the best studied model systems, Fur functions as a global regulator of iron homeostasis controlling both the induction of iron uptake functions (under iron limitation) and the expression of iron storage proteins and iron-utilizing enzymes (under iron sufficiency). We now appreciate that there is a tremendous diversity in metal selectivity and biological function within the Fur family which includes sensors of iron (Fur), zinc (Zur), manganese (Mur), and nickel (Nur). Despite numerous studies, the mechanism of metal ion sensing by Fur family proteins is still controversial. Other family members use metal catalyzed oxidation reactions to sense peroxide-stress (PerR) or the availability of heme (Irr).

Keywords Fur · metalloregulation · Metal homeostasis

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Introduction

Fur was first described as an iron-responsive repressor of iron-transport systems in *Escherichia coli* (Hantke 1981; Bagg and Neilands 1987a, b). The early studies of the *E. coli* Fur and its role have been summarized in several excellent reviews (Bagg and Neilands 1987a, b; Escolar et al. 1999; Hantke 2001; Braun 2003). Together, these early studies led to a clear and compelling model for Fur mediated repression of target genes under iron-replete conditions. This working model posits that the coordination of one Fe^{2+} per monomer enables the dimeric Fur protein to bind a specific 19 bp DNA sequence, called the ''Fur box'', within the promoter of the regulated genes. The affinity of Fur protein for Fe^{2+} is poised to allow accumulation of sufficient intracellular iron to activate essential iron-containing and iron-utilising enzymes (e.g. enzymes for heme and FeS cluster synthesis). However, when iron levels exceed those needed for metalloenzyme function, Fur represses further uptake and thereby helps prevent iron overload. Typically, the binding of iron-loaded Fur hinders the access of RNA polymerase resulting in the repression of downstream genes (Fig. 1; mechanism 1).

Numerous studies support the validity of this general model and it likely accounts for a significant fraction of the iron-dependent regulation effected by Fur. However, recent results indicate

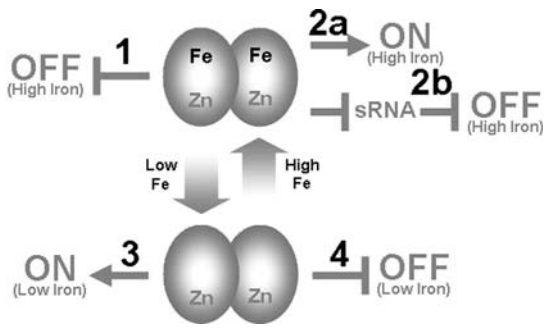

Fig. 1 Regulatory potential of bacterial Fur proteins. Fur proteins have been described to regulate gene expression by four general mechanisms. In most cases, it is the iron-loaded form of Fur protein (top) that binds to DNA operator sites. DNA-bound Fur can directly repress transcription (mechanism 1) or activate gene expression either directly or indirectly via small RNA molecules (mechanisms 2a and 2b, respectively). In *H. pylori*, the iron-free form of Fur also binds DNA and represses expression of an iron storage protein (mechanism 4). In the case of *B. japonicum* Irr, apo-protein binds DNA in the absence of a metal ion cofactor and can either activate (mechanism 3) or repress (mechanism 4) the expression of target genes (see Table 1 for a comparison among Fur family proteins). In general, when Fur functions as a repressor, the Fur box overlaps the promoter region, whereas activation is associated with a Fur box just upstream of the promoter. Note that Zur, Mur, and Nur regulate gene expression by mechanism 1 (although the regulatory metal ion is Zn^{2+}, Mn^{2+}, or Ni^{2+}, respectively, rather than Fe^{2+}). A structural Zn^{2+} ion is also shown in this example, although not all Fur proteins have a structural zinc (see text)

that regulation by Fur, and Fur-like proteins, can be much more complex. Specifically, we will focus here on: (i) current controversies regarding the site and consequences of regulatory metal ion binding to Fur family proteins, (ii) the diverse mechanisms used by bacterial Fur proteins to regulate their target genes, and (iii) the roles of Fur-like regulators that sense other metal ions, heme, and oxidative stress signals (Table 1).

Function of *Escherichia coli* Fur as a sensor of intracellular iron levels

The role of *E. coli* Fur (Fur_{EC}) as an Fe^{2+}-dependent repressor, as inferred from early genetic studies, was first demonstrated using an in vitro coupled-transcription translation system (Bagg and Neilands 1987a, b). In vitro, numerous other divalent cations can also activate the Fur protein to bind DNA. For reasons of convenience, the majority of studies employ Mn^{2+} (which unlike Fe^{2+} is stable in aerobic solutions) as a corepressor. The ability of Fur to bind DNA in response to Mn^{2+} likely underlies the observation that *fur* mutants have an increased ability to grow in the presence of elevated levels of Mn^{2+} (Hantke 1987). The implication is that excess Mn^{2+} in the growth medium can bind Fur and inappropriately repress iron homeostasis functions thereby impeding growth. Direct biochemical measurements have also provided evidence for a regulatory metal ion binding site with an apparent dissociation constant for metal ions in the low micromolar range (Mills and Marletta 2005). The affinity of Fur for ferrous iron is comparable to the estimated levels of free (loosely bound and chelatable) iron present in the cytosol (Keyer and Imlay 1996), consistent with a role for Fur as the primary monitor and regulator of intracellular iron levels. Presumably, occupancy of this regulatory metal-binding site alters the conformation of the protein to allow interaction with operator DNA.

Despite the apparent simplicity of this simple repression model, both the precise site of iron-binding and the effect on DNA-binding affinity are controversial. Fur_{EC} is presumed to contain a regulatory site for sensing Fe^{2+}. In addition, like many members of the Fur superfamily, Fur_{EC} contains a tightly bound structural Zn^{2+} ion that is required for proper protein folding (Jacquamet et al. 1998; Althaus et al. 1999). Fe^{2+} binds to Fur_{EC} in a site containing five or six N/O donor ligands, but the identity of the amino acid ligands are not well defined (Jacquamet et al. 1998). Biophysical analyses (such as X-ray absorption spectroscopy; XAS) suggest Zn^{2+} coordination in an $S_2(N/O)_2$ environment. Site-directed mutagenesis and chemical modification studies indicate that Cys residues in the $C_{92}XXC_{95}$ motif are essential for activity and function as direct Zn^{2+} ligands while those in the $C_{132}X_4C_{137}$ motif are dispensable (Coy et al. 1994; Gonzalez de Peredo et al. 1999). In the orthologous regulatory protein from *Bacillus subtilis* (Fur_{BS}), all four Cys residues in both CXXC motifs are essential for

Table 1 Representative fur family members

Protein subfamily	Organism	Structural/regulatory metal ion	Function(s)	Types of regulation[a]	Reference(s)
Fur	*E. coli*	Zn^{2+}/Fe^{2+} (Mn^{2+})	Fe uptake	1	Mills and Marletta (2005)
			Fe sparing	2b	Masse and Gottesman (2002
	B. subtilis	Zn^{2+}/Fe^{2+}	Fe uptake	1	Bsat and Helmann (1999)
			Fe sparing	2b	Our unpublished data
	P. aeruginosa	$Zn^{2+}?/Fe^{2+}$	Fe uptake	1	Pohl et al. (2003), Lewin et al. (2002) a
			Fe sparing	2b	Wilderman et al. (2004)
	N. meningitidis	$?/Fe^{2+}$	Fe uptake	1	Delany et al. (2004)
			Respiration	2a	Delany et al. (2004)
	H. pylori	$?/Fe^{2+}$	Fe uptake	1	Delany et al. (2001)
			Fe storage	4	Delany et al. (2001)
	B. japonicum	$-/Fe^{2+}$	Irr protein	1	Friedman and O'Brian (2004)
Zur	*E. coli*	Zn^{2+}/Zn^{2+}	Zn^{2+} uptake	1	Outten and O'Halloran (2001)
	B. subtilis	Zn^{2+}/Zn^{2+}	Zn^{2+} uptake	1	Gaballa and Helmann (1998)
			Zn^{2+} mobilization	1	Akanuma et al. (2006)
Mur	*R. leguminosarum*	$-/Mn^{2+}$ (Fe^{2+})	Mn^{2+} uptake	1	Diaz-Mireles et al. (2004)
Nur	*S. coelicolor*	$?/Ni^{2+}$	Ni^{2+} uptake	1	Ahn et al. (2006)
PerR[b]	*B. subtilis*	Zn^{2+}/Fe^{2+} (Mn^{2+})	Oxidative stress	1	Lee and Helmann (2006b)
			srf operon	2a	Hayashi et al. (2005)
	S. aureus	n.d./Mn^{2+} (Fe^{2+}?)	Oxidative stress	1(?)	Horsburgh et al. (2001)
Irr[c]	*B. japonicum*	-/Fe-heme	Fe^{2+} uptake	3	Hamza et al. (1998), Yang et al. (2006)
			Oxidative stress	4	Rudolph et al. (2006a, b)

[a] Types of regulation as described for Fur in Fig. 1 (with the indicated corepressor in place of Fe^{2+} where appropriate)

[b] PerR and its homologs require a metal as a corepressor and gene expression is induced by peroxide stress

[c] Irr binds DNA in the absence of a bound metal cofactor and can either activate or repress gene expression. Binding of heme triggers proteolysis of Irr (see text)

activity and protein stability in vivo (Bsat and Helmann 1999), and are likely to be involved in Zn^{2+}-coordination (our unpublished results). In contrast, some members of the Fur family appear to lack a structural Zn^{2+} ion (Lewin et al. 2002).

The publication of the crystal structure of *Pseudomonas aeruginosa* (Fur_{PA}) solved in complex with Zn^{2+} provided the first detailed view of the metal-binding sites in a Fur protein (Pohl et al.s 2003). The basic fold of the protein consists of two domains: an N-terminal DNA-binding domain and a C-terminal dimerization domain (Fig. 2). The winged-helix DNA binding domain of Fur_{PA} resembles that of the functionally analogous iron-sensor of *Corynebacterium diphtheriae*, DtxR, despite a lack of significant sequence similarity. On the other hand, the dimerization domain is different from that of DtxR, and does not resemble that of any known dimeric repressor. The structure of Fur_{PA} clearly identifies two metal-binding sites (Fig. 2B). Site 1 is located in the dimerization domain and the Zn^{2+} ion is coordinated by the side chains of His86, Asp88 (bidentate ligand), Glu107, His124, and by a water molecule showing a distorted octahedral geometry. Site 2 connects the DNA-binding domain and the dimerization domain, and comprises the side chains of His32, Glu80, His89, and Glu100 in a tetrahedral geometry. Fe^{2+} exchange experiments followed by XAS suggested that the Zn^{2+} ion at site 1, but not at site 2, is readily exchanged by Fe^{2+}. This led to the assignment of site 1 as the regulatory metal binding site and site 2 as a structural Zn^{2+}-binding site (Pohl et al. 2003).

The roles of sites 1 and 2, as visualized in the Fur_{PA} structure, are not yet clear. We suggest that site 1 (the ''iron'' site) may be a low affinity metal-binding site that is not essential for either protein function or sensing iron. There is little

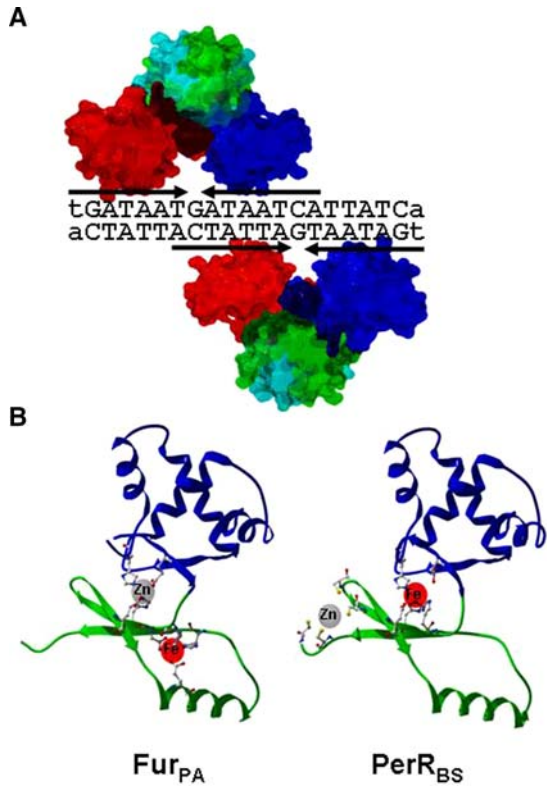

Fig. 2 Interaction of Fur family proteins with DNA and metal ions. (**A**) Model of the interaction of Fur_{PA} with DNA [Pohl et al. (2003) and Baichoo and Helmann (2002)]. The N-terminal DNA-binding domains are in red and blue, with the C-terminal dimerization domains in green. The classical Fur box is defined as a 19-bp inverted repeat sequence (capitals), originally assumed to bind one dimer. In this revised model of two overlapping 7-1-7 motifs (arrows), two Fur dimers bind the two 7-1-7 motifs from opposite side of the DNA [Baichoo and Helmann (2002)]. (**B**) Comparison of proposed metal-binding sites in Fur_{PA} and $PerR_{BS}$. In Fur_{PA} the structural Zn^{2+} (Site 2) is coordinated by side chains of His32, Glu80, His89, and Glu100 and connects the DNA-binding domain (blue) and dimerization domain (green) [Pohl et al. (2003)]. The regulatory Fe^{2+} is proposed to bind to Site 1 (occupied by Zn^{2+} in the crystal structure) located in the dimerization domain. In $PerR_{BS}$ (as modeled by homology with Fur_{PA}), the structural Zn^{2+} is coordinated by four thiolates (Cys96, Cys99, Cys136, and Cys139), locking the dimerization domain [Traore et al. (2006) and Lee and Helmann (2006a)]. The regulatory Fe^{2+}/Mn^{2+} site in $PerR_{BS}$ is predicted to include His37, Asp85, His91, His93, and Asp104, corresponding to site 2 in Fur_{PA}. PDB accession number of 1MZB was used for Fur_{PA} structure with replacement of Zn^{2+} at Site 1 with Fe^{2+}. The structure of $PerR_{BS}$ was generated by homology modeling based on Fur_{PA} (see also PDB accession number of 2FE3 for crystal structure of PerR:Zn)

evidence to suggest that this site is physiologically relevant. In contrast, mutational studies are consistent with the idea that the higher affinity ''zinc'' site (site 2) may in fact correspond to the iron-sensing site. For example, in Fur_{PA} Ala substitution mutations for two of the site 1 ligands (H86A and D88A) still retained iron-responsiveness, whereas an H89A mutant (a site 2 ligand) abolished iron-responsiveness (Lewin et al. 2002). The His residue corresponding to Fur_{PA} H89 is also critical for sensing iron in other Fur proteins including those from *B. subtilis* (H97A; Bsat and Helmann 1999), *Salmonella typhimurium* (H90R; Hall and Foster 1996) and *Vibrio cholerae* (H90L; Lam et al. 1994). Moreover, analysis of the *B. subtilis* Fur paralog, PerR, clearly demonstrates that ligands corresponding to site 2 are critical for binding Fe^{2+} but not for binding the structural Zn^{2+} ion (Lee and Helmann 2006a, b). Thus Fur_{PA} may sense iron through the site initially assigned as a candidate structural Zn^{2+} site (Fig. 2B). Indeed, as suggested by biochemical analyses (Lewin et al. 2002), Fur_{PA} may lack a structural Zn^{2+} ion.

Given the critical role of Fur as a sensor of intracellular iron levels, the Fur protein is surprisingly tolerant of mutations in candidate metal ligands. Mutational studies of Cys and His residues in Fur_{EC} demonstrated that only three residues were critical for in vivo function (Coy et al. 1994) and two of these (C_{92} and C_{95}) are now known to be ligands to the structural Zn^{2+} (Gonzalez de Peredo et al. 1999). The third, H_{32}, corresponds to a site 2 ligand in Fur_{PA} and may (by analogy with PerR) be one residue in the iron-sensing site. A similar phenomenon has been noted in mutagenesis studies with Fur_{BS}: most point mutations in likely iron ligands do not strongly affect iron regulation in vivo (our unpublished results). In *Bradyrhizobium japonicum*, a quadruple mutant with Ala substitutions for all four site 1 ligands still bound DNA in response to Mn^{2+} with an affinity only slightly (~2-fold) reduced relative to wild-type (Friedman and O'Brian 2004). Furthermore, a quadruple mutant of site 2 also bound DNA with near wild-type affinity, although in this case the ability to form a slower mobility complex (as observed in electrophoretic shift assays) was abolished.

Remarkably, both the site 1 and site 2 mutants were still able to repress gene expression in vivo (Friedman and O'Brian 2004).

In interpreting these results, it is important to consider the biological role of Fur as the central regulator of iron homeostasis. If a mutation weakens, but does not entirely eliminate, the affinity of Fur for iron, the resulting mutant cells may simply accumulate higher levels of intracellular iron (by virtue of their derepressed iron uptake pathways). As a result of elevated internal iron levels, the mutant Fur protein may still appear to respond normally. If correct, the mutant cells should have elevated levels of chelatable iron in their cytosol. However, even this rationalization fails to explain the results reported with *Bradyrhizobium japonicum* Fur in which a quadruple mutant of the presumed iron-sensing site could still respond to metal ions in vitro and in vivo (Friedman and O'Brian 2004). Clearly, it will be necessary to complement genetic analyses with biochemical measurements of iron affinity to resolve this conundrum.

Despite the confusion surrounding the precise location of the iron-sensing site in Fur proteins, it is still reasonable to suggest that iron-binding triggers repression by activating DNA-binding. However, several experiments challenge even this simple conclusion. For example, a second Zn^{2+} can bind to the presumed regulatory (Fe^{2+}-sensing) site of Fur_{EC}. Unexpectedly, both the Fur:Zn_1 and Fur:Zn_2 forms of Fur_{EC} were reported to bind DNA with high affinity (K_d ~20 nM) (Althaus et al. 1999). Similarly, Fur_{BS} bound DNA with high affinity in either the presence or absence of added Fe^{2+} (Bsat and Helmann 1999). These puzzling results beg the question: how can Fur respond selectively to Fe^{2+} in vivo if it binds DNA (at least in vitro) in both the presence and absence of an activating metal ion? The simplest explanation is that other metal ions in the experiments cited, either present as contaminants or as components of the buffers, were able to active Fur protein for DNA-binding. Recent results indicate that *E. coli* Fur:Zn_1 does not bind DNA if metal contamination is prevented by prerunning gels with EDTA to remove possible contaminating metal ions (Mills and Marletta 2005). In this study it was shown that Fur:Zn_1 could be activated to bind DNA by either Zn^{2+}, Co^{2+}, Fe^{2+}, or Mn^{2+}. Although both Zn^{2+} and Co^{2+} bound Fur with higher affinity than Fe^{2+}, comparison of the measured metal binding constants with estimated levels of intracellular metal ions suggest that only Fe^{2+} (and perhaps Mn^{2+}) are normally present at levels sufficient to effect repression (Mills and Marletta 2005). We note, however, that DNA-binding in the absence of an activating metal ion is not ruled out and, is physiologically relevant for some Fur family members (including *Helicobacter pylori* Fur and *B. japonicum* Irr; see below). It is embarassing to admit that 20 years after first being purified, the site of iron-sensing and the consequences of iron-binding, should be so poorly understood even for the founding member of this large protein family: Fur_{EC}.

Fur as a DNA-binding repressor

The mode of interaction of Fur with DNA has also been controversial (Escolar et al. 1999; Baichoo and Helmann 2002; Lavrrar and McIntosh 2003). Fur proteins generally bind to a 19-bp inverted repeat sequence known as a Fur box (GATAATGATwATCATTATC; w = A or T). Since Fur is a dimer, it was originally assumed that one dimer would bind to each Fur box and account for the typically observed 31 bp footprint. However, this model was not easily reconciled with detailed footprinting analyses and with the observation that Fur protein tends to polymerize at many operator sites to generate footprints that are not simple multiples of the 31 bp protected region. Indeed, Fur binds cooperatively at some promoter regions and generates helical arrays spiraling around the DNA duplex (Le Cam et al. 1994; Lavrrar et al. 2002).

These observations motivated detailed studies using synthetic oligonucleotides. As a result, Escolar et al. (1998) proposed a reinterpretation of the Fur box as three repeats of a 6 bp motif. According to this model, Fur binding requires three or more repeats of a simple 6 bp sequence in either direct or indirect repeats. However this interpretation is not easy to reconcile with the dimeric state of Fur and it is not clear whether

each 6 bp motif (NATWAT) represents the proposed binding site for one monomer or one dimer (Escolar et al. 1999).

Compilation and analysis of Fur regulated genes from *B. subtilis* led to a revised proposal (Baichoo and Helmann 2002). In this organism, some Fur regulated genes contained operator sites with only two of the previously noted 6 bp motifs embedded within a conserved 7-1-7 motif (tGATAATnATTATCa) (Baichoo et al. 2002). Moreover, operator sites recognized by other Fur paralogs in *B. subtilis* (Zur and PerR) also conform to this same general pattern (with one or two mismatches per half-site; Fuangthong and Helmann 2003). According to this model (Fig. 2A), the classic 19 bp Fur box is recognized by two Fur dimers, each interacting with one of two overlapping 7-1-7 motifs from opposite faces of the helix (Baichoo and Helmann 2002; Lavrrar et al. 2002). This type of interaction is reminiscent of the structure of DtxR bound to its operator sequence (White et al. 1998). Further extension of this model, to include 3, 4 or more Fur dimers, may account for the extended footprints observed at some operator sites (Lavrrar et al. 2002). Depending on the strength of the protein–protein interactions, the binding of Fur in such extended arrays might be expected to have a reduced requirement for a close match to the Fur binding consensus, once binding is nucleated at a high affinity site.

The presence of 19 bp Fur box consensus sequences is correlated with iron-repressible genes in numerous bacteria (Panina et al. 2001; Baichoo et al. 2002; Grifantini et al. 2003; Rodionov et al. 2004). In addition, Fur protein from many different sources can at least partially complement an *E. coli fur* mutant, providing further evidence that DNA-binding specificity is conserved among many family members. Indeed, simply searching bacterial genomes for close matches to the 19 bp Fur consensus identifies numerous candidates for iron-regulated genes. In the case of *B. subtilis*, approximately one-half of the Fur regulon could be identified by this simple expedient (Baichoo et al. 2002) and a similar correlation was reported for *Neisseria meningitidis* (Grifantini et al. 2003). This approach misses weaker sites and sites that match the shorter 7-1-7 consensus (and presumably bind only one dimer). In addition, recent evidence suggests that in some cases Fur and Fur-like proteins may recognize distinctly different classes of DNA-binding sites. These alternative sites may represent binding sites for non-metallated protein, for example (see below).

The ability to identify Fur-regulated genes by searching genomes for sites matching the Fur box consensus is further complicated in those organisms containing more than one Fur paralog. In *B. subtilis*, for example, sites recognized by Fur, Zur, and PerR are all quite similar (Fuangthong and Helmann 2003). Despite this similarity, there are no documented examples of sites that are regulated by more than one Fur-like protein binding to the same operator site (although such sites can be easily engineered; Fuangthong and Helmann 2003). There is no reason to believe, however, that such regulon overlap may not exist. Indeed, in *Campylobacter jejuni* both Fur and PerR appear to contribute to the repression of catalase in iron-containing medium (van Vliet et al. 1999).

Fur as a transcriptional activator

Fur_{EC} has long been implicated as both a positive and negative regulator of gene expression. Proteins that require Fur and iron for their efficient expression include iron superoxide dismutase (SodB), succinate dehydrogenase, and ferritin. Suggestive evidence for a positive regulatory role for Fur was also apparent in the early application of global approaches to analyzing the effects of Fur on gene expression. For example, proteomic analysis of *fur* mutants in *V. cholerae* revealed numerous proteins that apparently required Fur and iron for their expression (Litwin and Calderwood 1994).

It is now appreciated that positive regulation by Fur is often indirect, mediated by Fur-dependent repression of an anti-sense regulatory small RNA (sRNA) (Fig. 1, mechanism 2b). This RNA acts at the post-transcriptional level to repress translation of target genes (Masse and Gottesman 2002). The best characterized Fur-regulated sRNA, *E. coli* RyhB, is a 90 nt sRNA repressed

by Fur in an iron-dependent manner (Masse et al. 2005). Upon limitation for iron, or in a *fur* mutant, expression of RyhB is derepressed thereby causing a decrease in the level of target mRNAs. Target genes include the genes previously observed under the positive control of Fur, such as *acnA* (aconitase A), *fumA* (fumarase A), *sdhCDAB* (succinate dehydrogenase), *bfr* (bacterioferritin), *ftnA* (ferritin), and *sodB* (FeSOD). As a result of down-regulation of nonessential iron-containing enzymes and iron-storage proteins, *E. coli* is able to remodel its proteome to more efficiently use available iron for essential functions under iron limiting conditions (the ''iron-sparing response''). The characterization of RyhB and the iron-sparing response further emphasizes the role of Fur as a global regulator of gene expression (Abdul-Tehrani et al. 1999; McHugh et al. 2003; Masse et al. 2005).

The iron-sparing response is an extremely important, but only recently appreciated, aspect of iron homeostasis (Masse and Arguin 2005). sRNA-mediated iron-sparing pathways are present in other Gram negative bacteria (*Shigella* spp., *Vibrio* spp., and Pseudomonads; Wilderman et al. 2004; Mey et al. 2005; Payne et al. 2006) and a functionally similar system has recently been characterized in *B. subtilis* (our unpublished results). In the baker's yeast, *Saccharomyces cerevisiae*, an iron-regulated RNA-binding protein coordinates a similar response (Puig et al. 2005) and in *C. diphtheriae* the iron-dependent repressor DtxR represses a protein regulator of the iron-sparing response (Wennerhold et al. 2005). The independent evolution of ''iron-sparing'' responses in these diverse organisms attests to the importance of this process in adaptation to metal ion limitation.

Recent evidence suggests that some Fur proteins can also act as a direct transcriptional activator (Fig. 1 mechanism 2a). In *N. meningitidis*, Fur mediates classical iron-dependent repression of some genes (e.g. the transferrin receptor gene *tbp2*) while acting as an activator for others (e.g. *norB, pan1*, and *nuoABCDE;* Delany et al. 2004). Fur binds upstream of the promoters of these genes in a metal-dependent manner and, at least for *norB*, can directly activate transcription by RNA polymerase in vitro. Similarly, iron-loaded Fur appears to be an activator of *nifS* transcription in *H. pylori* (Alamuri et al. 2006).

Fur proteins and novel modes of regulation

The above results highlight the evolution, over the past few years, of our views of Fur. Once thought of as a simple, iron-dependent repressor, we now appreciate that Fur can be either a repressor or an activator. However, even in these cases, the DNA-binding activity of Fur requires bound iron. In some systems, however, Fur proteins can sense signals other than iron, and may bind DNA even in the absence of a bound metal cofactor.

Fur has been genetically implicated in various processes not obviously linked to iron homeostasis including acid tolerance (Foster and Hall 1992), high salinity response (Hoffmann et al. 2002), and chemotaxis (Ernst et al. 2005). One of the more intriguing findings, still unexplained, is the observation that an iron-blind mutant of Fur in *Salmonella enterica* still functions in the acid tolerance response (Hall and Foster 1996). It is not known, in this system, which target genes Fur binds in the absence of iron or how it effects regulation.

Evidence for Fur binding in the absence of iron has been most clearly documented in *H. pylori* (Fig. 1, mechanism 4). In this organism, the expression of ferritin is activated by iron in a Fur-dependent manner (Delany et al. 2001). Since expression is constitutive in a *fur* mutant, it is proposed that Fur binds as a repressor in the absence of bound iron, and derepression ensues when Fur binds Fe^{2+}. Support for this model was provided by in vitro DNA-binding studies which document the postulated iron-mediated decrease in DNA-binding affinity for this site (but not for sites repressed in the presence of iron) (Delany et al. 2001). A similar model has been advanced to explain the iron-mediated induction of *sodB*: apo-Fur binds the *sodB* promoter region, albeit with relatively low affinity, and binding is relieved in the presence of manganese (Ernst et al. 2005). The structural features that distinguish those sites bound by Fur in the absence versus the presence

of its iron cofactor have not been elucidated. The ability of Fur to bind to distinct sites in the presence and absence of iron may also play a role in the autoregulation of the *fur* gene by an ''anti-repression'' mechanism (Delany et al. 2003).

In addition to iron, Fur may also respond, at least in some cases, to reagents that bind or react with Fe^{2+}. Fur-repressed genes are at least partially derepressed in response to high levels of H_2O_2 in *B. subtilis* in some (Mostertz et al. 2004), but not other (Helmann et al. 2003), studies. Fur from several different organisms has also been reported to respond to nitric oxide (NO) or NO donors, most likely by direct nitrosylation of Fe^{2+} (D'Autreaux et al. 2002; Moore et al. 2004; Richardson et al. 2006).

Fur-family members that sense other metal ions (Zur, Mur, and Nur)

The Fur family of proteins is widespread within the Bacteria with ~800 homologs represented in the current compilations of the PFAM (PFO1475; 764 matches) and EMBL InterPro (IPR002481; 837 matches) databases. Approximately one half of these identified representatives are from the proteobacteria and another ~200 from the Firmicutes. The emerging consensus is that most of these proteins are likely to function as metal-dependent, DNA-binding repressors. However, within this family the metal-dependence and metal-specificity varies widely, even among the small minority of members that have been functionally characterized. Thus, it is important to appreciate that proteins annotated as ''ferric uptake repressor,'' or with a related descriptor, may or may not actually sense iron. Examples of Fur family members that respond to other metals include sensors of zinc (Zur), manganese (Mur), and nickel (Nur).

Zur: sensors of zinc starvation

The ability of Fur family members to function physiologically as sensors of Zn^{2+} was discovered concurrently in *B. subtilis* (Zur_{BS}) and *E. coli* Zur (Zur_{EC}) (Gaballa and Helmann 1998; Patzer and Hantke 1998). Subsequently, genomic analyses have allowed tentative assignments of likely Zur regulons in numerous other bacteria (Panina et al. 2003). Zur_{EC} represses the expression of an ABC-type Zn-specific uptake system (ZnuACB) by binding to the bidirectional promoter region of *znuA* and *znuCB*. When cells are starved for zinc, derepression of this high affinity uptake system aids in zinc acquisition (Patzer and Hantke 2000). In *B. subtilis*, Zur_{BS} was discovered in the course of functional characterization of the multiple Fur paralogs encoded in genome (Gaballa and Helmann 1998). In response to zinc sufficiency, Zur_{BS} represses a zinc uptake ABC transporter (encoded by the *ycdHI-yceI* operon) and a complex operon encoding proteins of largely unknown function (*yciABC*) (Gaballa et al. 2002). Although initially the YciABC system was postulated to function as low affinity zinc-uptake system or permease (Gaballa et al. 2002), recent data suggest a different role for these proteins under zinc limited conditions. YciA is an alternate enzyme for folate biosynthesis (V. de Crecy-Lagard, personal communication) that can substitute for the Zn^{2+}-dependent FolE(MtrA) protein (our unpublished data). YciC is a putative metallochaperone which is postulated to allow metal insertion into YciA.

Zur also represses several genes encoding homologues of ribosomal proteins (Panina et al. 2003). Under zinc-deficient conditions, for example, the *B. subtilis ytiA* gene (a non-zinc-containing L31 paralog) is derepressed, and the newly synthesized YtiA replaces RpmE (zinc-containing ribosomal protein L31) (Akanuma et al. 2006). Since ribosomes are highly abundant in rapidly growing cells, the presence of two or three zinc-containing ribosomal proteins represents a large reservoir of zinc and may in fact account for the majority of intracellular zinc. It is postulated that the released L31 (and another zinc containing ribosomal protein, L33) is degraded to supply cells with zinc. Thus, like Fur, Zur may function as a global regulator to control not only metal ion uptake, but also metal ion storage and mobilization.

Biochemically, Zur_{EC} is the best characterized member of the Zur sub-family of regulators (Outten and O'Halloran 2001; Outten et al. 2001). Like Fur_{EC}, Zur_{EC} contains two distinct

metal-binding sites with different affinity and coordination environment. Zur containing only one Zn^{2+} ion per monomer (Zur:Zn_1) does not bind DNA. In the presence of excess Zn^{2+}, Zur (presumably Zur:Zn_2) can bind to the *znuC* promoter and sterically hinders the binding of RNA polymerase (Outten and O'Halloran 2001). The structural Zn^{2+} ion in Zur:Zn is tightly bound and difficult to replace with other metal ions. The coordination environment of this Zn^{2+} ion has been determined to be S_3(N/O) by XAS, and is proposed to involve two Cys residues in the $C_{103}XXC_{106}$ motif by analogy with Fur_{EC}. The second metal binding site in Zur_{EC} can bind either Zn^{2+} or Co^{2+} and is readily exchangeable. The geometry of this regulatory site was assigned as tetrahedral $S(N/O)_3$ by combined XAS and UV-vis spectroscopy studies using Zur:Zn,Co (Outten et al. 2001). The apparent affinity of Zur_{EC} for Zn^{2+}, as determined using TPEN as a Zn^{2+} buffer, was $1\sim2 \times 10^{-16}$ M: a value indicating that there is no free zinc in the cell at equilibrium (Outten and O'Halloran 2001). It remains to be determined whether or not other Zur homologs have a similar high affinity.

Mur: the manganese-uptake regulator

The Fur homolog in *Rhizobium leguminosarum* represses the transcription of the ABC-type Mn^{2+} transporter *sitABCD* (*mntABCD*) operon under manganese replete conditions, but not under excess iron conditions (Diaz-Mireles et al. 2004). Thus, this protein was named Mur (manganese uptake regulator). Mur_{RL} apparently lacks a structural Zn^{2+} binding site and instead binds two Mn^{2+} ions per dimer with micromolar dissociation constants (Bellini and Hemmings 2006). Activated Mur_{RL} binds to a unique binding site (MRS; Mur responsive sequence) distinct from canonical Fur boxes (Diaz-Mireles et al. 2005). However, Mur_{RL} partially regulates the *bfd* gene (having a canonical Fur box) in an Fe-dependent manner in an *E. coli fur* mutant background. In vitro, Mur_{RL} can also use Fe^{2+}, Co^{2+}, or Zn^{2+} as activating metals, and the consequent metallated Mur binds to both MRS and canonical fur boxes as one and two homodimers (Bellini and Hemmings 2006). Thus, Mur may in fact have an overlapping DNA-recognition specificity with Fur. The ability of Mur to respond selectively to Mn^{2+} (in *R. leguminosarum)* and to Fe^{2+} (in *E. coli*) is a reminder that many metalloregulators will bind a fairly broad range of metal ions (as confirmed in in vitro binding studies), but their in vivo responsiveness is dictated by the available levels of metals in the cytosol. Similar results were noted when the iron-specific repressor DtxR was expressed in *B. subtilis* (Guedon and Helmann 2003), and in studies of metalloregulators of the ArsR/SmtB family (see Tottey et al. 2005 for a discussion).

Nur: the nickel-uptake regulator

Recently, a nickel-uptake regulator (Nur) was discovered in *Streptomyces coelicolor* thereby adding a nickel responsive member to the Fur family (Ahn et al. 2006). Nur exhibits significant similarity to other members of Fur family having 27% identity (48% similarity) to Fur_{EC}. Nur has four of the five conserved amino acids predicted to constitute the regulatory metal-sensing site (based on functional studies of PerR; Lee and Helmann 2006b), with the fifth replaced by His. This is the likely location for a Ni^{2+}-selective regulatory site (J.-H Roe, personal communication). In addition, both CXXC motifs that comprise the structural Zn^{2+}-site in $PerR_{BS}$ (see below) are well conserved in Nur. In contrast, the predicted regulatory metal site inferred from Fur_{PA} is poorly conserved with only one match out of four.

Nur negatively regulates the FeSOD gene *sodF* and the putative nickel-transporter gene cluster *nikABCDE*, by binding to promoter regions in the presence of nickel (Ahn et al. 2006). Although the expression of NiSOD encoded by *sodN* is induced by the presence of nickel, Nur does not appear to be a direct activator for *sodN*, nor does it seem to use a sRNA-mediated mechanism. Unlike many other Fur family proteins (e.g. Fur, Mur, and PerR), which can be activated by several metal ions in vitro, Nur is highly specific for Ni^{2+} both in vitro and in vivo.

Fur-family members that sense other signals (PerR, Irr)

Evolution has apparently adapted the basic scaffold of the Fur repressor family for sensing signals other than metal ions. In the case of PerR, the peroxide-regulon repressor first characterized in *B. subtilis* (Chen et al. 1995; Bsat et al. 1998), the iron-binding site now functions as a metal-based sensor of peroxides (Lee and Helmann 2006b). *B. japonicum* Irr (iron-responsive regulator) binds DNA in the absence of bound ligand to regulate numerous iron- and heme-related genes (Rudolph et al. 2006a, b; Yang et al. 2006a, b).

PerR and its homologs

PerR (peroxide regulon repressor) is the major regulator of the inducible peroxide stress response in *B. subtilis* and is the prototype for a group of related peroxide sensing repressors found in both Gram-positive and Gram-negative bacteria (Bsat et al. 1998; Mongkolsuk and Helmann 2002). The PerR regulon includes MrgA (a Dps-like DNA-binding protein; Chen et al. 1993; Chen and Helmann 1995), the major vegetative catalase (KatA; Chen et al. 1995), alkyl hydroperoxidase (AhpCF; Bsat et al. 1996), enzymes of heme biosynthesis (HemAXCDBL; Chen et al. 1995), a zinc uptake P-type ATPase (ZosA; Gaballa and Helmann 2002), and both the PerR and Fur metalloregulatory proteins (Fuangthong et al. 2002). Most PerR-regulated genes are derepressed in cells treated with low level of H_2O_2 or simultaneously starved for both iron and manganese (Fuangthong et al. 2002). Physiological and biochemical studies indicate that PerR contains a structural Zn^{2+} and can be activated to bind DNA by either Fe^{2+} or Mn^{2+} as corepressor thereby generating two forms of PerR (PerR:Zn,Fe or PerR:Zn,Mn) which differ in their sensitivity to peroxide (Herbig and Helmann 2001; Lee and Helmann 2006a, b).

The candidate ligands for metal-binding in PerR were predicted by homology modeling and their roles tested by site directed mutagenesis (Lee and Helmann 2006b). The regulatory (Fe^{2+} or Mn^{2+}) binding site includes His37, Asp85, His91, His93, and Asp104 (corresponding to the putative Zn^{2+} site in Fur_{PA}). Ala substitution mutants of these residues still bind Zn^{2+}, but no longer act as functional repressors in vivo (despite the elevated protein levels). Both CXXC motifs of PerR cluster in the three-dimensional structural model to create a candidate structural Zn^{2+} binding site (Lee and Helmann 2006b). Mutations of these residues lead to unstable protein in vivo. Furthermore, oxidation of these residues leads to disulfide bond formation and loss of Zn^{2+} (Lee and Helmann 2006a). Thus, PerR contains a Cys_4:Zn structural site and a histidine-rich regulatory metal binding site. The recent determination of the crystal structure of apo-PerR:Zn confirms the presence of the predicted Cys_4:Zn site and reveals a splayed protein conformation in which the two DNA-binding domains are projecting in opposite directions from the dimeric protein core (Traore et al. 2006). It is postulated that a large (160°) domain rotation is necessary to bring His37 (from the DNA-binding domain) into proximity with the other regulatory site binding ligands.

Recent biochemical studies have established that PerR uses a novel mechanism for H_2O_2 sensing. Reaction of Fe^{2+}, bound to the regulatory site, leads to a metal-catalyzed oxidation (MCO) of the protein (Lee and Helmann 2006b). Physiologically relevant (micromolar) levels of H_2O_2 lead to the rapid oxidation of either His37 or His91, two of the candidate ligands for the regulatory metal site. Oxidation is postulated to lead to 2-oxo-Histidine formation and a weakening of the metal affinity of the regulatory site. In vivo, oxidation of either residue appears sufficient to elicit derepression.

This MCO mechanism for peroxide-sensing contrasts with most other characterized peroxide-sensors which instead use reactive protein thiolates (Paget and Buttner 2003; Kiley and Storz 2004). Examples include the OxyR and OhrR families of proteins. Although it was initially hypothesized that Cys residues might be involved in peroxide sensing by PerR, the oxidation rate of the Cys_4:Zn site (~0.05 $M^{-1}s^{-1}$) is too slow to account for in vivo peroxide sensing (Lee and Helmann 2006a). Furthermore, in vivo studies indicate that no detectable Cys oxidation occurs

even in the presence of 10 mM H_2O_2 (Lee and Helmann 2006a). Thus, in contrast to thiol-based peroxide sensors (such as OxyR, RsrA, and Hsp33), PerR does not use Cys thiolates for peroxide sensing.

PerR and PerR-like regulators have been described in a wide variety of organisms. In *Staphylococcus aureus* PerR controls a regulon of oxidative stress resistance and iron storage proteins including many of those described in *B. subtilis* (Horsburgh et al. 2001; 2002). $PerR_{SA}$ appears to be a Mn^{2+}-specific repressor in vivo and exhibits induction of the PerR regulon by elevated iron concentrations (Horsburgh et al. 2001; Morrissey et al. 2004). It is not clear whether or not Fe^{2+} can function as a corepressor in vitro (these studies would likely require anaerobic conditions) .These results may simply indicate that under the growth conditions used the rate of oxidative inactivation of $PerR_{SA}$ by peroxides was sufficient to lead to derepression of the iron form even in the absence of added H_2O_2 (the PerR:Zn,Mn form of the *B. subtilis* protein is at least 10^6-fold less sensitive to peroxide-inactivation than the PerR:Zn,Fe form). In the catalase-negative organism *Streptococcus pyogenes*, PerR is the only Fur homolog (King et al. 2000). Although PerR mediates the inducible peroxide resistance response and represses the *mrgA* gene involved in peroxide-resistance, AhpC and GpoA (glutathione peroxidase) are regulated in PerR-independent manner (King et al. 2000). Furthermore, it has been postulated that PerR may be involved in some functions of Fur including repression of iron-hydroxymate transporter, FhuADBG operon (Brenot et al. 2005). In *Streptomyces coelicolor*, CatR regulates the CatA (the major vegetative catalase A) protein (Hahn et al. 2000). PerR has also been described in *Listeria monocytogenes* (Rea et al. 2004, 2005). PerR homologs are not as prevalent in the Gram negative bacteria. However, PerR orthologs are clearly present in the Gram-negative pathogens *C. jejuni* (van Vliet et al. 1999) and *Helicobacter hepaticus* (A. van Vliet, personal communication). *C. jejuni* lacks OxyR and $PerR_{CJ}$ regulates catalase and alkyl hydroperoxide reductase in response to iron and oxidative stress (van Vliet et al. 1999).

Irr: a Fur-based heme sensor

Bradyrhizobium japonicum Irr was discovered as a protein required for the iron-activated expression of the heme biosynthesis enzyme, δ-aminolevulanic acid dehydratase (Hamza et al. 1998). In addition, an *irr* mutant strain was deficient in expression of high affinity iron uptake suggesting a more general role in coordinating iron homeostasis (Hamza et al. 1998). The mechanism of regulation of Irr is unusual within the Fur superfamily: Irr functions as a DNA-binding protein under low iron conditions and is rendered inactive by rapid proteolytic degradation in the presence of iron (Qi et al. 1999). The actual effector of protein degradation is not free iron, but heme delivered to a heme-binding site on Irr via a direct interaction with ferrochelatase (Qi and O'Brian 2002).

Functional analysis of the Irr protein reveals the presence of two heme-binding sites involved in iron-dependent regulation (Yang et al. 2005). The first is a classical heme-recognition motif near the amino-terminus and binds ferric heme (Qi et al. 1999). The second site binds ferrous heme and includes a cluster of three His residues that align with the H91 and H93 ferrous iron ligands in *B. subtilis* PerR. Like PerR, regulation of Irr has recently been shown to involve oxidative modification of the protein and protein oxidation requires this ferrous heme binding site (Yang et al., 2006a). Thus, Irr monitors iron availability by sensing the ability of ferrochelatase to synthesize heme from protoporphyrin IX and iron and may also sense oxidative stress.

Recent results have linked Irr to regulation of genes by the iron-control element (ICE) associated with numerous iron-regulated genes (Rudolph et al. 2006a, b; Yang et al., 2006b). Interestingly, Irr appears to act as either a repressor or an activator of gene expression under low iron (heme) conditions. Heme biosynthesis genes are repressed under low iron conditions and induced by the heme-dependent degradation of Irr. Conversely, genes involved in iron uptake are repressed by iron and this repression requires both Irr and heme. The presence of ICE sequences suggests that Irr binds directly to many of these promoters and activates

transcription, and degradation of Irr in the presence of heme and oxygen leads to a loss of transcription.

In addition to Irr, *B. japonicum* also harbors a classical Fur protein which functions to regulate expression of *irr* and the *hemA* gene in response to iron (Hamza et al. 1999). Interestingly, the binding site recognized by Fur_{BJ} differs significantly from the canonical Fur box (Friedman and O'Brian, 2003). Mutational studies of iron-regulation by Fur_{BJ} demonstrated that this activity did not require candidate amino acid ligands from either site 1 or site 2 as defined in the FurPA crystal structure (Friedman and O'Brian 2004). This suggests that this protein, like Irr, may have a novel mode of iron recognition.

Concluding remarks

The Fur family of metalloregulatory proteins display a remarkable variety of regulatory mechanisms. While it is true that the majority of characterized Fur family members can be thought of as iron-dependent, DNA-binding repressors, this simple model does not capture the full range of regulatory potential. In addition to iron, metal-dependent repression mechanisms have been described for proteins that sense zinc (Zur), manganese (Mur), and nickel (Nur). Other family members employ metal-catalyzed protein oxidation to regulate DNA-binding activity and thereby respond physiologically to the presence of peroxides (PerR) or heme and oxygen (Irr). In addition to repressing gene expression, Fur family members can act directly to stimulate transcription from target promoters and, in several systems, regulate gene expression indirectly through small RNA or protein intermediaries. While here we have emphasized the regulatory potential of Fur-like proteins, it is also clear that they often function as global regulators of metal homeostasis and genomic technologies are allowing rapid progress in defining the often extensive regulons controlled by these proteins.

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