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MINIREVIEW

Meddling with Metal Sensors: Fur-Family Proteins as Signaling Hubs

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ABSTRACT The ferric uptake regulator (Fur) protein is the founding member of the FUR superfamily of metalloregulatory proteins that control metal homeostasis in bacteria. FUR proteins regulate metal homeostasis in response to the binding of iron (Fur), zinc (Zur), manganese (Mur), or nickel (Nur). FUR family proteins are generally dimers in solution, but the DNA-bound complex can involve a single dimer, a dimer-of-dimers, or an extended array of bound protein. Elevated FUR levels due to changes in cell physiology increase DNA occupancy and may also kinetically facilitate protein dissociation. Interactions between FUR proteins and other regulators are commonplace, often including cooperative and competitive DNA-binding interactions within the regulatory region. Further, there are many emerging examples of allosteric regulators that interact directly with FUR family proteins. Here, we focus on newly uncovered examples of allosteric regulation by diverse Fur antagonists (*Escherichia coli* YdiV/SlyD, *Salmonella enterica* EIIA^{Ntr}, *Vibrio parahaemolyticus* FcrX, *Acinetobacter baumannii* BlsA, *Bacillus subtilis* YlaN, and *Pseudomonas aeruginosa* PacT) as well as one Zur antagonist (*Mycobacterium bovis* CmtR). Small molecules and metal complexes may also serve as regulatory ligands, with examples including heme binding to *Bradyrhizobium japonicum* Irr and 2-oxoglutarate binding to *Anabaena* FurA. How these protein-protein and protein-ligand interactions act in conjunction with regulatory metal ions to facilitate signal integration is an active area of investigation.

KEYWORDS metal homeostasis, transcription, metalloregulation, allosteric regulation, repressor, activator

Iron is an essential element in all domains of life and is used as a cofactor in numerous enzymes that are required for metabolism and growth (1). When iron levels are low, the enzymes that are required for central metabolism, respiration, and DNA synthesis may fail and thereby limit growth. Conversely, high concentrations of iron (Fe^{2+}) accelerate reactions with hydrogen peroxide (H_2O_2) (via the Fenton reaction), which produces toxic hydroxyl radicals that damage DNA and proteins (2, 3). Thus, it is crucial that iron be maintained at optimal levels within the cell (4, 5). Fur (ferric uptake regulation protein) was first purified from *Escherichia coli* in 1987 and was found to be an iron-activated repressor (6). Unlike many DNA-binding regulators, Fur is abundant, with an estimate of 10,000 proteins per cell in *E. coli* (7), which suggests that Fur may additionally serve as an iron buffer. Fur is now recognized as the founding member of the FUR superfamily, a diverse set of metal-regulated transcription factors (8). Here, we briefly summarize the role of FUR proteins in the metal-dependent regulation of gene expression, a topic that has been covered in more detail in several recent reviews (9–13). Then, we focus on the ability of diverse proteins and small molecules to allosterically regulate FUR protein function, often in response to yet unknown signals.

MODEL FOR REGULATION BY FUR FAMILY PROTEINS

Fur is a homodimeric protein containing at least one iron-sensing site on each protomer (3, 10). Each Fur protomer may additionally have a structural zinc site that is

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important for protein folding (14, 15). In some cases, a secondary metal-sensing site may be present (10, 16). At low ambient levels of cytosolic ferrous iron (Fe^{2+}), the regulatory metal-sensing site is unoccupied, and the dimeric Fur protein is in an "open" conformation that is not conducive to DNA-binding. The binding of Fe^{2+} to the regulatory site generates a "closed" protein conformation in which the two metal-binding domains are appropriately positioned for high-affinity DNA-binding. The precise conformation changes, and the molecular details of metal-binding and selectivity have been visualized for several Fur family members and are reviewed elsewhere (10). This simple model, in which Fur functions as an iron-dependent transcriptional repressor, accounts for most Fur regulation, as characterized using genome-wide methods (17–25). Because cytosolic pools of Fe^{2+} are affected by the oxygen tension in the cell, Fur can also function indirectly as a sensor of O_2 (24).

FUR family proteins include the ferric uptake regulator (Fur) (6), zinc sensor Zur (12), nickel sensor Nur (26, 27), manganese sensor Mur (28–30), and their orthologs (sometimes with different names). Each of these metalloregulators senses changes in ambient metal levels to regulate metal ion homeostasis (see [9] for a recent review). In addition, members of the PerR (peroxide stress) subfamily of regulators sense H_2O_2 stress rather than metal status (reviewed in [11]). The prototype for this group, namely, *B. subtilis* PerR, senses H_2O_2 when the oxidation of bound Fe^{2+} generates a hydroxyl radical that covalently modifies a proximal histidine side chain, thereby leading to protein inactivation (31, 32) and, ultimately, degradation (33). Bound ferrous ions can also be nitrosylated, leading to the derepression of Fur and PerR-regulated genes in response to nitric oxide (34, 35). Orthologs of PerR are widespread in bacteria, including many pathogens, although there are variations in the precise signals that are sensed, the biochemical mechanisms of sensing, and the suite of genes under PerR regulation (11).

Although many FUR regulators are specific for a single cognate metal ion *in vivo*, biochemical studies reveal that DNA binding can also be activated by noncognate metal ions. Indeed, the ability of Mn^{2+} to mismetalate Fur proteins and thereby inappropriately repress iron acquisition likely accounts for the ability of mutations in *fur* to suppress Mn^{2+} intoxication in several systems (36–38). This type of regulatory crosstalk can be aggravated by mutations that perturb metal ion homeostasis, often with deleterious consequences, as noted for *B. subtilis* (39–41). For PerR subfamily proteins, the abilities of both Fe^{2+} and Mn^{2+} to activate DNA binding allows these proteins to tune their H_2O_2 sensitivity in response to the Fe/Mn ratio in the cell (41).

Although FUR proteins typically act as classic transcriptional repressors, other regulatory modes are possible, including transcriptional activation by the metalated regulator and regulation by the DNA-bound apo-protein (42). Direct transcriptional activation by DNA-bound FUR proteins was first documented for *Neisseria meningitidis* Fur (43) and *Helicobacter pylori* Fur (44–46). A direct activation mechanism has also been documented for *E. coli* Fur (25), *B. subtilis* Fur (47), *Vibrio cholerae* Fur (48), *Campylobacter jejuni* Fur (49), *Streptomyces coelicolor* Zur (50), and *Xanthomonas campestris* Zur (51). Many organisms contain multiple FUR proteins, and the DNA-binding sites are often similar in sequence (9, 52). Despite this, regulatory crosstalk in which one binding site is recognized by more than one FUR protein is rare, although some examples have been noted (47, 53). FUR binding may, at some sites, also be regulated by epigenetic DNA modifications, such as adenine methylation (54).

Fur proteins may also indirectly activate gene expression via the repression of negatively acting small RNAs (sRNA). In turn, these sRNAs reduce the translation of abundant iron-containing proteins and iron-storage proteins, thereby allowing Fur to indirectly activate their expression when iron is abundant. This type of regulation is generically referred to as an iron-sparing response (reviewed in references [55, 56]). Though rarer, apo-Fur can also act as a direct activator, as documented in *E. coli* (25), *C. jejuni* (22), and *Staphylococcus aureus* (57). *Borrelia burgdorferi* BosR may also be an example of a Fur homolog that functions as an activator in the absence of a regulator metal (although it requires a structural Zn ion for protein folding) (58).

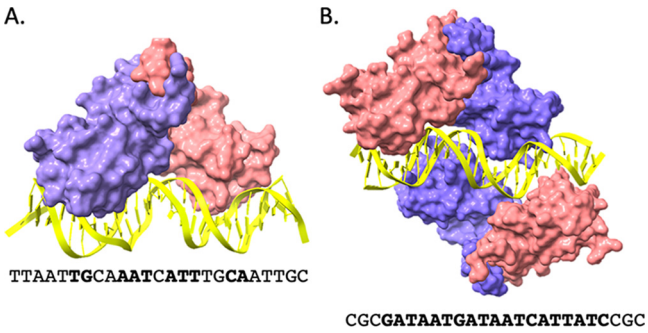

FIG 1 Space-filling representation of Fur protein (from *Magnetospirillum gryphiswaldense* MSR-1) (62) that was activated by Mn^{2+} and bound to operator DNA. (A) A dimer of Fur protein (with one protomer in purple and another in pink) bound to the *P. aeruginosa feoAB1* operator site (PDB: 4RB3). Bases that match the 7-1-7 consensus (TGATAATnATTATCA) for Fur binding are in bold. (B) Two Fur dimers in complex with a consensus 19 bp Fur box (PDB: 4RB1), which can be represented as two overlapping 7-1-7 consensus sites (61).

Homotypic interactions: oligomerization and polymerization of FUR proteins. Functioning as a traditional repressor, iron-bound Fur (holo-Fur) binds to DNA to prevent the transcription of genes involved in iron acquisition and in the iron-sparing response (17–25, 59). As originally defined, the Fur consensus (Fur box) is a 19 bp inverted nucleotide repeat in DNA (60). This sequence is best viewed as two overlapping 15 bp (7-1-7) inverted repeats (61). At such sites, two Fur dimers interact with overlapping repeat sequences on opposing faces of the DNA (Fig. 1), as visualized in structures of the *Magnetospirillum gryphiswaldense* Fur protein bound to DNA (62) and in the structure of *E. coli* Zur, which is a zinc-sensing Fur paralog (63). This general architecture is conserved across bacteria, and searches using a modified information theory model of overlapping Fur-binding sites was successful at the identification of Fur-regulated genes (64). A similar dimer-of-dimers model likely applies to other members of the extended FUR superfamily (65). FUR proteins can also regulate genes when bound as a single dimer, as when *B. subtilis* Fur represses the expression of the *feuA* promoter controlling ferri-siderophore uptake functions (66).

Although FUR proteins are commonly observed as stable dimers in solution, other oligomeric states may also be relevant. For example, purified Fur proteins from *Pseudomonas aeruginosa*, *Francisella tularensis*, and *Legionella pneumophila* form stable tetramers, and tetramer dissociation is required to generate dimers that are competent to bind DNA (67, 68). Similarly, *Anabaena* FurC (a PerR ortholog) forms both noncovalent and disulfide-linked tetramers in solution (69).

At some regulatory regions, FUR dimers may oligomerize to form extended arrays or interact to form compacted nucleoprotein structures (70, 71). The extended complexes formed by Fur may result from contiguous DNA-binding sites or result from favorable protein-protein interactions that are nucleated by an initially bound Fur dimer. Other FUR family members can also form extended arrays on DNA. The precise arrangement of the oligomerized FUR proteins along or around the DNA helix has not been resolved. *Streptomyces coelicolor* Zur has been studied in detail, and repression was associated with Zur binding to target sites as dimers or tetramers, whereas activation of the *zitB* efflux gene was associated with the formation of an extended Zur protein array (50). FUR proteins may also bind cooperatively to spatially separated sites. For example, *H. pylori* Fur forms a repression complex at the *arsRS* acid acclimation operon with as many as three separated binding sites condensed together to form a compacted nucleoprotein complex. In sum, FUR proteins may bind as dimers, as a dimer-of-dimers (Fig. 1), or in more extensive complexes, including extended protein arrays or compact complexes that are mediated by DNA looping or wrapping.

In addition to increasing DNA occupancy, dimers from solution may kinetically facilitate protein dissociation from DNA (Fig. 2) (72). This type of protein-assisted DNA dissociation can be observed via the single-molecule tracking of fluorescently labeled

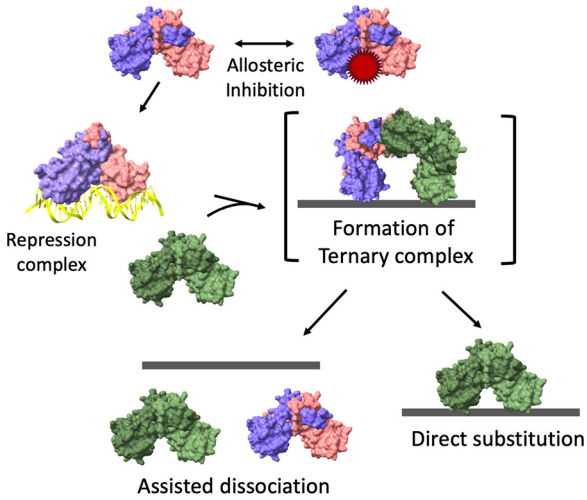

FIG 2 Schematic illustration of a representative Fur family protein binding to DNA as a dimer and the regulatory impact of allosteric regulators and protein-facilitated operator dissociation. Most antagonists likely act from solution to impede the binding of the metalloregulator to DNA (gray line). Once bound, protein still in solution is postulated to interact transiently with DNA-bound protein to form an unstable ternary complex that can then resolve to yield either a direct substitution (with no change in regulation) or the dissociation of both dimers (leading to either derepression or a loss of activation), as described in (72–74).

proteins *in vivo* by monitoring the on and off rates of DNA-bound complexes as a function of protein concentration. In the first noted example, it was found that the increasing concentrations of the *E. coli* CueR metalloregulator (a member of the MerR family) led to an increase in the DNA dissociation rate (73). Similarly, *E. coli* Zur dissociates from its operator site in a concentration-dependent manner that is indicative of protein-assisted dissociation (74). The ability of metalloregulators to kinetically facilitate protein dissociation may be particularly important for those regulators that bind metal ions, such as Cu^{+} and Zn^{2+}, which bind with high affinity to proteins. These ions can have low rates of dissociation from their cognate metalloregulators, and, when the dissociation of bound protein is also slow, this can create a kinetic barrier to gene induction in response to the falling levels of metals in the cell.

Heterotypic interactions: competition and cooperation in metalloregulation. FUR proteins, like most transcription factors, can also be affected by interactions with other regulators that bind in the vicinity of their cognate operator sites or even compete for the same sites. The binding of multiple regulatory proteins may be independent, cooperative, or competitive. Transcription regulation is thereby dependent on the precise set of bound proteins, which allows the integration of multiple signals to control gene expression.

Competition often results when regulatory binding sites are positioned to allow one protein to sterically exclude another. This type of interaction is seen in the competitive binding of a Mn^{2+}-responsive Fur homolog (Mur) and an iron-responsive regulator Irr in the regulatory region of the *irr* gene in *Bradyrhizobium japonicum* (75, 76). At this promoter, Mur represses transcription, and Irr acts as an antirepressor under iron limitation by binding to an overlapping operator site and occluding Mur binding. A similar type of antagonism has been suggested to affect DNA occupancy by *B. subtilis* Fur, depending on the activity of two other DNA-binding regulators that signal redox changes in the cell: ResD and NsrR (77). ResD activity is correlated with anaerobiosis, whereas NsrR signals the presence of nitric oxide, a reactive gas that antagonizes Fur function via the nitrosylation of bound iron (34, 35). Similarly, the DNA binding of the *H. pylori* Fur protein can be antagonized by NikR, a Ni-responsive repressor (78, 79). While direct competition for a single binding site is rare, in the regulatory region controlling the expression of the *B. subtilis pfeT* gene (encoding an iron efflux ATPase), Fur

and PerR compete for binding at one of three DNA-binding sites (47). This type of overlapping DNA recognition reflects the fact that the *B. subtilis* Fur, PerR, and Zur consensus sites differ by only one or two bases per half-site. At the *pfeT* regulatory region, PerR binds cooperatively to two upstream sites with the promoter proximal site overlapping the promoter. The competitive binding of Fur to the promoter distal site allows for the replacement of the PerR repression complex with the Fur protein, which, at this promoter, functions as a transcription activator (47).

Cooperative binding between FUR proteins and other DNA-binding regulators has also been observed. The *B. subtilis catDE* operon, encoding a catechol dioxygenase, is repressed by Fur, and this repression is cooperative with CatR, which is a sensor of catechols (19). Similarly, NsrR and ResD bind cooperatively with Fur at some coregulated sites under anaerobic conditions (77). In *S. aureus*, the synthesis and uptake of the nicotianamine-like metallophore staphylopine is regulated by both Fur and Zur (53). In this case, the biosynthesis gene cluster (*cntK* operon) is regulated independently by Fur and Zur from separate binding sites. However, the uptake genes (*cntA* operon) are apparently regulated by the cooperative binding of both Fur and Zur. This uptake operon is most sensitively repressed by low levels of Zn, but this repression requires both the Fur and Zur proteins (53). Thus, FUR proteins can bind competitively or cooperatively with other regulators, a property shared with many other DNA-binding transcription factors.

PROTEIN-PROTEIN INTERACTIONS THAT ANTAGONIZE DNA BINDING

In addition to cooperative and competitive interactions between FUR proteins and other DNA-binding regulators, numerous examples have emerged of allosteric regulatory proteins that bind FUR proteins to prevent DNA binding. In the simplest case, a regulator binds a FUR protein to form an inactive complex, but more complex types of interactions are also likely involved.

Uropathogenic *E. coli*: modulation of Fur activity by YdiV and SlyD. Uropathogenic *E. coli* (UPEC) infections require the successful navigation of environments with highly variable iron availability. During the early stages of infection, the bacteria require flagellar motility to colonize the host, whereas at later stages, flagellar motility is turned off to evade the host's innate immune system, and high-affinity iron uptake is induced (80). Recently, the EAL (Glu-Ala-Leu) domain protein YdiV and SlyD, which is a peptidyl-prolyl cis-trans isomerase, were found to function together to reduce DNA binding by Fur in UPEC (81). The *ydiV* gene is itself induced in response to the low iron conditions, but this induction is independent of Fur. In a strain overexpressing YdiV, the DNA-binding activity of Fur decreases 300-fold, and this decrease depends on the presence of the SlyD prolyl isomerase. Fur protein purified from cells with YdiV and SlyD is in an altered conformation, and this conformational change depends on a Pro residue at position 18. A bacterial two-hybrid study revealed formation between both Fur-SlyD and Fur-YdiV, but not between SlyD-YdiV. Thus, a model emerges in which YdiV binds to Fur, and this may enhance the ability of the SlyD prolyl isomerase to convert Fur into an inactive conformer (Fig. 3). It is not yet clear whether this isomerization occurs only during the folding of nascent Fur protein or whether it might act on extant Fur molecules. The two conformers, presumably differing in the presence of a cis- versus trans-Pro bond, still bind iron with similar affinities, but they differ greatly in their ability to bind DNA. The two Fur conformers also differ in their propensity to form disulfide bonds, but it was not established whether a disulfide bond occurs *in vivo*; disulfide bonds in cytoplasmic proteins are generally rare and transient. Together, YidV and SlyD are important for the efficient expression of iron uptake functions, and, therefore, for establishing an infection within host bladder epithelial cells (81). In addition to its role in helping counter the Fur repression of iron uptake functions, YdiV also functions as a protein antagonist of the major activator of flagellar motility, namely, the $FlhD_4C_2$ protein complex. This regulator may thereby help coordinate the induction of iron uptake functions with the shutoff of flagellar motility during the invasion into epithelial cells.

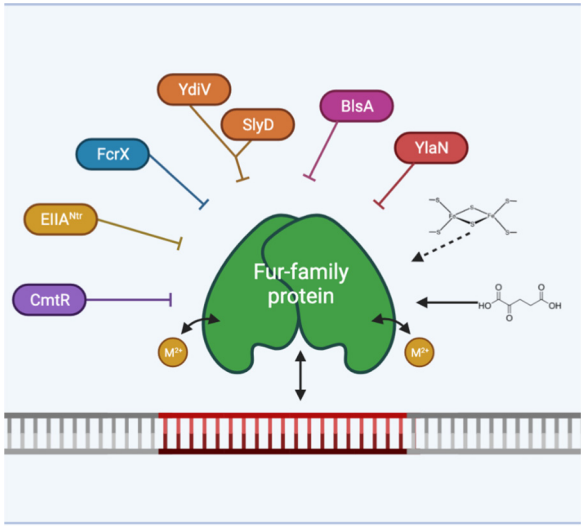

FIG 3 Summary of protein and small molecule antagonists that affect the function of FUR regulators. FUR regulators most commonly require a divalent metal ion (M^{2+}) to activate DNA-binding. However, numerous other factors have now been revealed that can also modulate FUR function, often by acting as antagonists of DNA-binding. The signals that control the expression and activity of each of these effectors are only partially understood (see the text for details).

The details of how the YdiV-SlyD system is itself regulated remains to be clarified. Although *ydiV* is induced by low iron conditions, the nature of this regulation is unclear. Further, whereas many EAL domain proteins bind and hydrolyze cyclic-di-GMP, the YdiV family of standalone EAL domain proteins are catalytically inactive and do not bind c-di-GMP (82). The activity of SlyD is also puzzling, as it is not yet clear whether it only acts during protein folding to affect the activity of newly synthesized Fur protein, nor is it established whether the reported effects of protein conformation on disulfide bond formation are relevant *in vivo*.

***Salmonella enterica*: EIIA^{Ntr} antagonizes Fur DNA binding.** *Salmonella enterica*, like many bacteria, employs phosphotransferase (PTS)-dependent sugar import systems that couple the phosphorylation of sugars to their import. This system is initiated via the phosphoenolpyruvate (PEP)-dependent phosphorylation of enzyme I (EI) followed by phosphotransfer to histidine protein (HPr), membrane tethered EIIA and EIIB proteins, and, ultimately, to sugars being imported into the cell (83). In addition, several proteobacteria encode a parallel nitrogen phosphotransferase system (PTS^{Ntr}) that comprises a homologous set of proteins: EI^{Ntr}, Npr, and EIIA^{Ntr} (84). The EI^{Ntr} protein binds L-glutamine and 2-oxoglutarate (2-OG) to integrate information about cellular nitrogen and carbon fluxes. The output of the PTS^{Ntr} system is phosphorylated EIIA^{Ntr}, which interacts with a variety of proteins to allosterically regulate their activity (85).

Iron homeostasis is of paramount importance during infection of the mammalian host, which is often an iron-restricted environment. Choi and Ryu have shown that EIIA^{Ntr} regulates iron uptake genes through a protein-protein interaction with Fur (86). *S. enterica* encounters iron limitation within the macrophage phagosome. In response, *S. enterica* derepresses Fur-regulated iron uptake systems. However, the derepression of the Fur regulon is compromised in *ptsN* mutant strains that lack the EIIA^{Ntr} protein (87). Interestingly, this regulatory effect of EIIA^{Ntr} is dependent on the presence of Fur, suggesting that EIIA^{Ntr} may physically interact with Fur to decrease iron uptake repression. Indeed, a bacterial two hybrid assay revealed a physical interaction between EIIA^{Ntr} and Fur, and, *in vitro*, EIIA^{Ntr} antagonizes Fur binding to DNA.

There are over 600 species of bacteria that contain both Fur and EIIA^{Ntr} proteins, and, thus, EIIA^{Ntr} may affect iron homeostasis in other related organisms (86). Because EIIA^{Ntr} is involved in regulating other cellular processes, it is possible that EIIA^{Ntr} regulation may allow Fur to integrate signals besides iron starvation. Intriguingly, phosphorylation was

not necessary for the interaction of $EIIA^{Ntr}$ and Fur (86). Thus, it is unlikely that nitrogen or glutamine concentrations are the key signals for this $EIIA^{Ntr}$-dependent regulation. Instead, $EIIA^{Ntr}$ may function by helping titrate free Fur protein to assist in the derepression of the Fur regulon under low iron conditions. A mechanism for the titration of free metalloregulator, and possibly for protein-assisted dissociation, may be particularly important for Fur, as this protein can be abundant, with up to 10,000 proteins per cell in *E. coli* (7).

***Vibrio parahaemolyticus:* FcrX antagonizes Fur DNA binding.** Iron can be a limiting nutrient for many marine species, including the marine pathogen *Vibrio parahaemolyticus*, which exists as both swimming cells in marine environments as well as swarming cells during the colonization of surfaces, such as the chitinous shells of crustaceans (88). *V. parahaemolyticus* differentiates from unipolarly to laterally flagellated cells on surfaces, thereby allowing for swarming motility and colonization. Differentiation into swarmer cells requires a temporal gene expression cascade that is mediated by the LafK activation of σ^{54}-depedent promoters. This differentiation is inhibited by polar flagella and is induced by iron limitation.

Morabe and McCarter discovered that the *fcrX* gene is repressed by Fur in iron-replete conditions (89). In a *fur* mutant, or upon overexpression of *fcrX*, cells exhibit increased swarming motility and derepress iron uptake. Together, this suggests that FcrX plays an antagonistic role toward Fur. Indeed, FcrX binds directly to Fur, as judged by coimmunoprecipitation evidence. The authors propose a model in which Fur binds iron under iron-sufficient conditions and represses the transcription of genes containing an upstream Fur box (90). As iron concentrations fall, the expression of Fur-regulated genes, including *fcrX*, is increased. FcrX then binds to Fur to block iron loading or to inhibit repression by holoFur. Alternatively, FcrX might work to kinetically facilitate the dissociation of holoFur from DNA. Further studies are needed to discern how Fur regulation is integrated with the LafK-dependent activation of swarming-related genes (91–94). Since swarmer cell differentiation genes lack Fur boxes, iron regulation may be indirect (90). One possibility is that Fur regulates the expression of another regulator that is required for the expression of these swarmer cell genes.

***Acinetobacter baumannii*: photoreceptor BlsA allows for the light-sensitive modulation of the Fur regulon.** In the multidrug resistant human pathogen *Acinetobacter baumannii*, light intensity influences biofilm formation, metabolism, antibiotic resistance, virulence, and iron limitation responses (95). Blue light using flavin (BLUF) proteins sense blue light and transmit this signal to downstream effector proteins through protein-protein interactions. The *A. baumannii* BLUF photoreceptor BlsA antagonizes the Fur-dependent repression of gene expression in the dark and at 23°C but not at 30°C (96). Consistently, even under iron-limited conditions, the expression of the Fur-regulated acinetobactin siderophore was only induced in the dark and in a *blsA*-dependent manner. The BLUF protein BlsA allows both light and temperature signals to regulate *A. baumannii* Fur when the organism is outside the host. How these light and temperature signals may affect the sensing of iron status during infection is not yet clear.

***B. subtilis:* YlaN modulates Fur DNA binding.** *B. subtilis* Fur represses a large regulon of genes involved in siderophore biosynthesis and iron import pathways, and it indirectly activates numerous Fe-containing enzymes through the small RNA FsrA (97). The transcription of *fur* is repressed by another FUR protein, namely, the peroxide-sensor PerR. In a *perR* null mutant, Fur protein levels are elevated by approximately 2.2-fold, and this increase results in the repression of iron uptake functions due to the binding of intracellular Mn^{2+} to the Fe^{2+}-sensing sites in Fur (40). This inappropriate repression of iron uptake contributes to a severe growth defect in *perR* mutant strains. Indeed, *perR* is essential in *B. licheniformis*, unless *fur* is also inactivated (98). These findings highlight the importance of tightly regulating Fur protein levels. This also illustrates the deleterious consequences of the mismetalation of metalloregulatory proteins. For example, Mn^{2+} can act as a toxic agonist of Fur proteins in many Gram-negative organisms.

Genome-wide efforts in *B. subtilis* led to the identification of approximately 260 essential genes (99). Among these, several have no functionally characterized homologs,

and these were therefore considered to be attractive candidates for the development of new antimicrobials. One such gene, namely, *ylaN*, encodes a small, dimeric protein that is comprised of 3 alpha-helices (100). Using a CRISPRi inhibition approach to characterize the consequences of depletion, Peters et al. discovered that the severe growth defects due to *ylaN* depletion (and even deletion) could be rescued by iron supplementation (101). In a genome-wide screen of protein-protein interactions that were detected using a bifunctional cross-linker, YlaN was found to bind to Fur protein, with the cross-linked lysine residue in Fur (K74) being near the regulatory metal-binding site (102). Thus, YlaN is postulated to bind to and inhibit Fur, and, in the absence of YlaN, an unregulated increase in Fur-mediated repression and subsequent iron starvation may account for the apparently essential role of *ylaN*. YlaN proteins are largely restricted to Bacilli and relatives (COG4838), and the gene seems to be constitutively expressed. It is not yet clear which signals may regulate the amount or activity of YlaN in the cell to modulate iron homeostasis.

***Mycobacterium bovis* CmtR antagonizes Zur DNA binding.** Mycobacterial species encode up to five paralogous type VII secretion systems (T7SS), and they are known as ESX-1 through ESX-5 (103, 104). The ESX-3 system is intimately linked to metal homeostasis, as judged by the inability of the *Mycobacterium tuberculosis* mutants in this locus to grow in culture, unless supplemented with iron or, even better, iron and zinc (105). The ESX-3 system secretes effector proteins that are important for siderophore-mediated iron acquisition (106), and they may additionally be involved in other metal homeostasis pathways. Indeed, the expression of the ESX-3 locus is repressed by three different metalloregulators: the iron sensor IdeR (107), the zinc sensor Zur (108), and the manganese sensor MntR (109).

The *M. bovis* ESX-3 system is additionally regulated by CmtR, which is a metalloregulatory protein of the SmtB/ArsR family (110). CmtR likely functions physiologically as a thiol-dependent sensor of redox stress (111). In response to peroxide stress, CmtR dissociates from an autoregulatory binding site in its own promoter region, thereby leading to an increase in CmtR protein. Unexpectedly, CmtR enhanced the expression of the ESX-3 T7SS, despite having no binding activity to the promoter region of this operon. This effect results from binding between CmtR and Zur, which reduces Zur DNA binding. These results support a model in which oxidative stress derepresses *cmtR* expression and elevated CmtR antagonizes the Zur repression of the *esx3* operon (111).

Products of the *esx3* operon, including EsxG and EsxH, help combat oxidative stress, although the mechanism is still unclear. EsxGH may act as external zinc scavengers that chaperone zinc to importers. Such external zinc chaperones have been identified in *B. subtilis* (ZinT) and in *P. dentrificans* (AtzD). It has been suggested that Zn import may help alleviate oxidative stress, but the molecular basis for this effect is not clear. In fact, H_2O_2 can lead to the demetallation of Fe-dependent enzymes that can then be inactivated via metalation by Zn (112). Under these conditions Zn import might be disadvantageous. Further efforts are needed to clarify how these multiple regulators bind to the *esx3* promoter region (Zur, IdeR, MntR) and, acting from solution (CmtR), work together to sense diverse signals to control ESX3 expression.

***Pseudomonas aeruginosa* PacT inhibits Fur DNA binding.** Toxin-antitoxin (TA) systems in bacteria consist of a stable toxin protein and a cognate unstable antitoxin. The bacterial pathogen *Pseudomonas aeruginosa* encodes multiple type II TA systems, which are those with a toxin that is regulated by a protein antitoxin (113). One recently characterized type II TA system, namely, PacTA, contains a toxin (PacT) that has a GCN5-related N-acetyltransferase (GNAT) domain and is thought to arrest translation via the acetylation of charged tRNAs. The cognate anti-toxin, namely, PacA, is a member of the AraC family of DNA-binding proteins (113). In addition to its effects on translation arrest, the PacTA system also affects iron homeostasis. A proteomics analysis of *pacTA* deletion mutants revealed that several Fur-regulated iron-uptake proteins had reduced expression, suggesting that PacTA modulates iron homeostasis in *P. aeruginosa* (114). PacT specifically bound to *P. aeruginosa* Fur *in vitro* to inhibit DNA binding. The inhibition of Fur DNA-binding activity by PacT derepresses genes for iron uptake

and biofilm formation, two traits that are important for *P. aeruginosa* virulence. Interestingly, the coexpression of Fur and PacT reduced the toxic effect of PacT, suggesting that Fur binding to PacT also provides some antitoxin activity. In addition, *pacT* and *pacA* expression is enhanced upon the addition of the iron chelator dipyridine, indicating that iron starvation acts as a signal for the induction of the PacTA system via a mechanism that is still unknown. Together, these results support a model in which PacT inhibits bacterial translation, unless it is bound to Fur or attenuated by the antitoxin PacA.

SMALL MOLECULES THAT AFFECT FUR FAMILY PROTEIN FUNCTION

***Bradyrhizobium japonicum* Irr: regulation by the binding of heme.** In alphaproteobacteria, the Irr subfamily of FUR proteins sense iron in the form of the biologically important chelate heme. The best studied representative of this subfamily is *Bradyrhizobium japonicum* Irr (9, 115). *B. japonicum*, like many other alphaproteobacteria, also encodes Mur, which is a FUR protein that senses Mn^{2+}. In turn, Mur regulates the expression of Irr, which responds physiologically to changes in iron status (76). Despite its role in regulating iron homeostasis, Irr (like Mur) binds DNA in complex with Mn^{2+} (115). Indeed, Irr binds Mn^{2+} with high affinity, suggesting that Mn^{2+} is the relevant metal cofactor for DNA binding under all but the most Mn^{2+}-limited conditions. Mn^{2+} binds to Irr in a site resembling the metal activation site in *B. subtilis* PerR, which also binds to Mn^{2+} with a higher affinity (K_d of approximately 2.8 μM) (15) than does the dedicated Mn^{2+} sensor MntR (K_d of approximately 13 μM) (116). Under iron replete conditions, heme synthesized by ferrochelatase is delivered to Irr, where it competitively displaces Mn^{2+}, thereby antagonizing DNA binding (117). An additional layer of regulatory control results from the proteolytic instability of the heme-bound Irr protein. The displaced Irr-heme complex can be oxidized and degraded, which further reinforces the derepression of Irr-regulated genes. The heme-dependent release of Irr from DNA leads to the expression of heme synthesis enzymes, iron-containing proteins, and proteins involved in iron storage and efflux. Irr is also an activator of transcription, and, when heme levels are low, Irr binds DNA to activate the expression of iron uptake genes (76). At sites where Irr functions as an activator, heme may antagonize activation, independent of DNA dissociation or degradation (118). The picture that emerges is that Irr senses heme rather than free Fe^{2+} to monitor iron status, and it does so by competition with Mn^{2+}. Together, Mur and Irr coordinate gene expression and allow cells to integrate signals that are related to Mn^{2+}, Fe^{2+}, redox status, and heme availability.

***Anabaena* sp. PCC 7120: 2-oxoglutarate enhances DNA binding by Fur at some sites.** The extent to which FUR proteins regulate gene expression through interactions with other small molecules is not yet clear. In one example, *Anabaena* sp. PCC 7120 FurA senses both Fe^{2+} and 2-oxoglutarate (2-OG) (119), which is a central metabolite at the intersection of the Krebs cycle (carbon metabolism) and nitrogen metabolism. The accumulation of 2-OG serves as a signal of nitrogen limitation. FurA binds to 2-OG with a high affinity (K_d of approximately 2.8 μM) (119), and this allows FurA to repress *ntcA*. NtcA is a CRP (cAMP receptor protein) family protein that regulates nitrogen scavenging and assimilation genes, and it is important for the differentiation of nitrogen-fixing heterocysts.

The FurA regulation of *ntcA* appears to be part of a complex feedback loop. The binding of 2-OG to NtcA activates nitrogen assimilation genes and also upregulates the expression of FurA. FurA also senses 2-OG and represses *ntcA*, thereby completing a feedback loop to shut off further NtcA synthesis. The repression of *ntcA* by FurA requires a bound metal cofactor (Mn^{2+} is used for *in vitro* studies), and it is enhanced when 2-OG is present. In contrast, 2-OG did not affect FurA binding to a different target site, namely, *isiB* (119). Thus, nitrogen assimilation genes can be regulated by NtcA and FurA, and they are affected by iron and redox homeostasis as well as nitrogen limitation (120).

FurA may also sense other signals, in addition to Fe^{2+} and 2-OG. Some FUR regulators have a Cys-rich metal-binding site that coordinates a Zn^{2+} ion that is necessary for proper protein folding. Although FurA has two CxxC motifs, the protein does not

copurify with Zn^{2+}. The FurA Cys residues are redox active and can be found in either reduced or disulfide bond form in the cytosol (121). The C-terminal CxxC motif is maintained in a reduced state by thioredoxin A (TrxA) (122), and one of these Cys residues (C141) contributes to the ability of FurA to bind heme, which prevents DNA binding (123–125). How redox status may regulate FurA activity *in vivo* is not fully resolved, but only the fully reduced form of FurA is competent to sense 2-OG and to respond to heme.

***E. coli* Fur: iron-sulfur cluster assembly.** *E. coli* Fur is one of the best characterized metalloregulators, and it has both a structural Zn^{2+} site and an Fe^{2+}-sensing regulator site. In addition, there is a third potential metal-binding site that is rich in Cys residues. Recently, it has been noted that *E. coli* Fur also has the potential to bind an iron-sulfur cluster [2Fe-2S] at this auxiliary sensing site (126, 127). The *in vivo* relevance of the [2Fe-2S] cluster assembly on Fur function is not yet clear. Only a small fraction (approximately 4%) of Fur protein purified from wild-type *E. coli* was found to contain a [2Fe-2S] cluster. However, upon the deletion of two iron-sulfur cluster assembly proteins, namely, IcsA and paralogous SufA, intracellular iron concentrations were significantly increased, and Fur was purified in a red form with approximately 31% of the protein containing a bound [2Fe-2S] cluster at site 3 (126). Site 3 is an auxiliary site of variable (and often uncertain) relevance that is found in a subset of Fur family members (10, 16). The third site contains three cysteine residues that, upon mutation, abolish the [2Fe-2S] cluster binding to Fur. Additionally, Fur homologs from *H. influenzae* and *Vibrio cholerae* can also bind substoichiometric levels of an [2Fe-2S] cluster when expressed in *E. coli* mutants with elevated intracellular iron levels (126, 128). To date, no evidence has been presented to demonstrate that the [2Fe-2S] cluster assembly impacts DNA binding or the expression of genes in the Fur regulon, nor is it clear whether the [2Fe-2S] cluster assembly is relevant in wild-type cells.

Conclusions. FUR proteins are ubiquitous in bacteria and most commonly function to sense divalent metal ions (including zinc, manganese, iron, and nickel) to regulate metal ion homeostasis. Here, we have focused on emerging insights into the ability of FUR proteins to integrate signals via protein-protein or small molecule-protein interactions (Fig. 3). While the demonstration that a diverse set of proteins can function as antagonists of metalloregulator activity is an important first step, additional efforts are required to better define how these allosteric regulators are themselves controlled and to discern the molecular messages that are represented by their binding. Small molecules also have regulatory potential, as is most carefully defined for heme and the 2-OG regulation of FurA in *Anabaena*, but here, too, there are many complexities that still remain to be explored. Metal ions are central to cell physiology, and metalloregulatory proteins and riboswitches provide the most direct measurement of intracellular metal status. Therefore, it is fitting that metalloregulators serve as such central regulatory hubs, controlling not only metal homeostasis but also diverse metabolic pathways, developmental programs, and virulence gene expression.

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