



Redox Sensing by Fe²⁺ in Bacterial Fur Family Metalloregulators

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Abstract

Significance: Iron is required for growth and is often redox active under cytosolic conditions. As a result of its facile redox chemistry, iron homeostasis is intricately involved with oxidative stress. Bacterial adaptation to iron limitation and oxidative stress often involves ferric uptake regulator (Fur) proteins: a diverse set of divalent cation-dependent, DNA-binding proteins that vary widely in both metal selectivity and sensitivity to metal-catalyzed oxidation.

Recent Advances: Bacteria contain two Fur family metalloregulators that use ferrous iron (Fe²⁺) as their cofactor, Fur and PerR. Fur functions to regulate iron homeostasis in response to changes in intracellular levels of Fe²⁺. PerR also binds Fe²⁺, which enables metal-catalyzed protein oxidation as a mechanism for sensing hydrogen peroxide (H₂O₂).

Critical Issues: To effectively regulate iron homeostasis, Fur has an Fe²⁺ affinity tuned to monitor the labile iron pool of the cell and may be under selective pressure to minimize iron oxidation, which would otherwise lead to an inappropriate increase in iron uptake under oxidative stress conditions. Conversely, Fe²⁺ is bound more tightly to PerR but exhibits high H₂O₂ reactivity, which enables a rapid induction of peroxide stress genes.

Future Directions: The features that determine the disparate reactivity of these proteins with oxidants are still poorly understood. A controlled, comparative analysis of the affinities of Fur/PerR proteins for their metal cofactors and their rate of reactivity with H₂O₂, combined with structure/function analyses, will be needed to define the molecular mechanisms that have facilitated this divergence of function between these two paralogous regulators. *Antioxid. Redox Signal.* 29, 1858–1871.

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Iron's Double-Edged Sword

IRON IS AN ESSENTIAL ELEMENT across all domains of life. It is used as a cofactor by enzymes involved in a wide array of metabolic processes spanning central metabolism, respiration, photosynthesis, nitrogen fixation, and DNA synthesis and repair (6). Bacteria are no exception to this, as is evident by their use of three major classes of iron-requiring proteins: (i) mono- and dinuclear iron enzymes, (ii) iron/sulfur cluster enzymes, and (iii) heme proteins. These three classes of iron-utilizing enzymes permeate many aspects of bacterial metabolism.

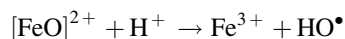
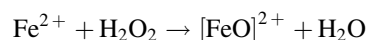
This need for iron in bacteria is ancient and dates from the earliest stages in the evolution of life >2 billion years ago. During this time, the Earth was an anoxic environ-

ment, which allowed iron to be maintained in its reduced, soluble ferrous (Fe²⁺) form (5). Iron was therefore highly bioavailable and found use as a cofactor for stabilizing proteins as “iron rivets” (39), for electron transfer reactions, and as a Lewis acid catalyst. However, as photosynthetic organisms became more prevalent, the gradual oxygenation of the Earth's atmosphere imposed a transition from anaerobic to aerobic conditions. This shifted the redox state of iron into its oxidized, weakly soluble ferric (Fe³⁺) form and led to the geologic deposition of massive amounts of iron in banded iron formations (5). These environmental changes imposed two new physiological challenges on early microbial life: iron starvation and oxidative stress.

The restriction on microbial growth that resulted from the depletion of dissolved iron from the ancient oceans persists to this day (97). As a result, bacteria evolved systems for iron homeostasis, which are often regulated by ferric uptake regulator (Fur) proteins (74). Iron homeostasis involves three distinct mechanisms (Fig. 1). First, bacteria synthesize high-affinity iron chelators (siderophores), their cognate uptake systems, and other transporters for iron import (6). Second, Fur often controls an “iron-sparing” response in which a Fur-regulated small RNA serves to inhibit the translation of nonessential iron requiring enzymes (82). Third, cells may replace iron-containing proteins with alternative, iron-independent proteins (87). For example, under iron limitation, many bacteria express flavodoxins as a substitute for iron-utilizing ferredoxins and manganese superoxide dismutase (MnSOD) in place of FeSOD (87). Collectively, these responses provide the cell with a viable iron economy despite the scarcity of the growth-limiting micronutrient.

The second chemical consequence of the rise of diatomic oxygen in the atmosphere was an increase in reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and su-

peroxide radicals (O_2^-). ROS are naturally generated in the environment in a variety of photosynthetic reactions and as metabolic by-products of flavoprotein autoxidation (62, 84). ROS can exert toxicity in several distinct ways, but one common theme is the oxidative inactivation of iron-containing enzymes, including both iron/sulfur-containing dehydratases and mononuclear iron enzymes (63). H_2O_2 reacts with ferrous iron through Fenton chemistry, and the resulting highly reactive hydroxyl radical can impair metabolism through DNA and protein damage, which can inevitably lead to cell death (63).



Similarly, superoxide inactivates iron-containing enzymes by oxidation of their required cofactors. In the case of mononuclear iron enzymes, oxidation and loss of iron have been shown to lead to mismetallation by zinc (Zn^{2+}), which

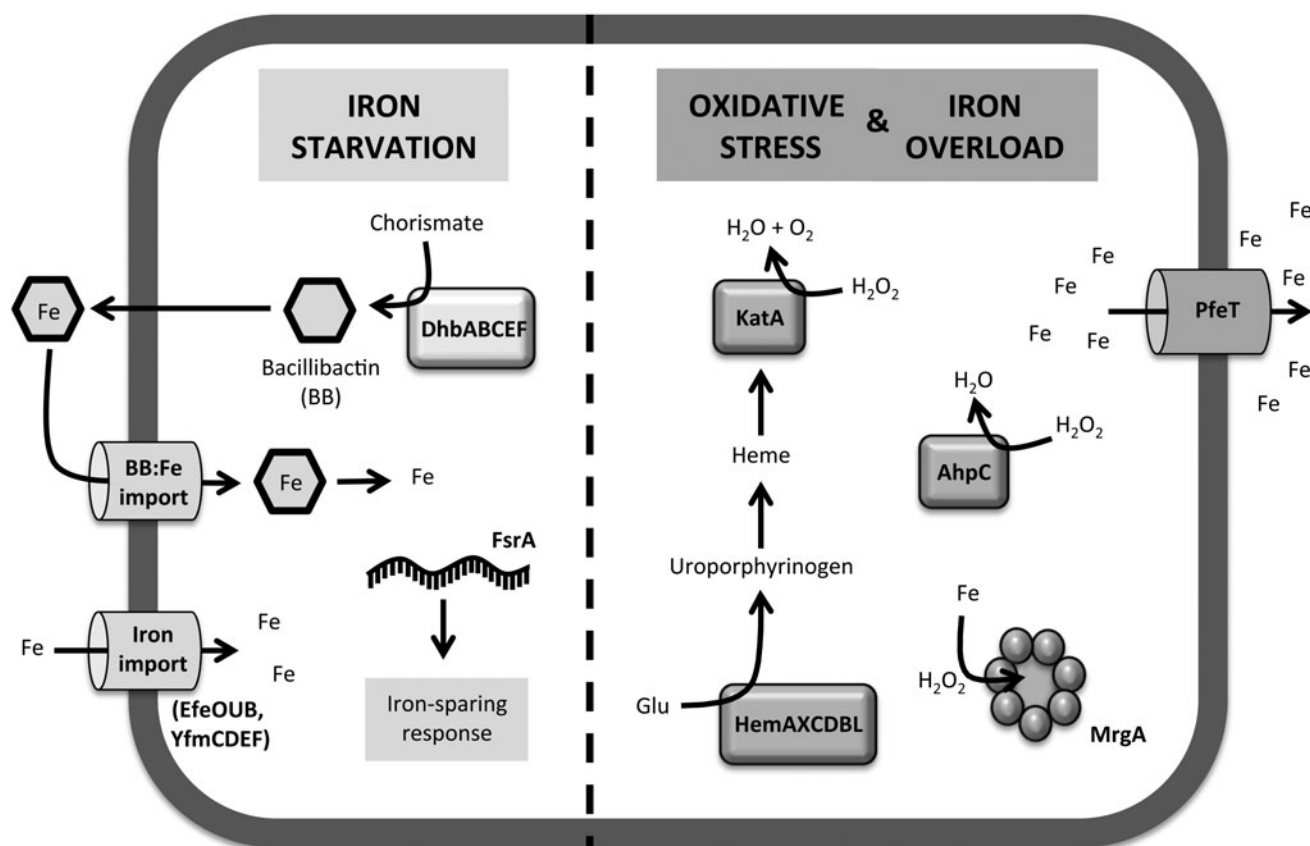


FIG. 1. Iron-related stress responses in *Bacillus subtilis*. Schematic summary of the response mechanisms in *B. subtilis* to different types of iron-related stresses. When cells are subjected to iron starvation, cells express genes involved in iron acquisition, which encode iron importers and siderophore biosynthesis enzymes. Cells also express the small RNA FsrA, which targets post-transcriptional inhibition of low-priority iron requiring enzymes, thus implementing the iron sparing response. Under conditions of oxidative stress, cells induce expression of peroxide detoxifying enzymes (KatA and AhpC) and their cofactors (HemAXCDBL), as well as enzymes that have an impact on cellular iron levels, such as the iron sequestration ferritin-like protein (MrgA) and the P_{1B_4} -type ATPase iron efflux pump PfeT. Note that under iron overload conditions, PfeT and (to a lesser extent) MrgA also confer resistance to excess iron. AhpC, alkyl hydroperoxide reductase; Fe, iron; KatA, catalase.

binds with higher avidity than iron (64). This has been observed in *Escherichia coli*, where ribulose-5-phosphate 3-epimerase and 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase become mismetallated by Zn^{2+} on exposure to ROS, causing enzyme inactivation and subsequent blockage of specific metabolic pathways (64, 104, 105).

Aerobic bacteria are reliant on detoxification mechanisms to help counter the deleterious effects of ROS, by either directly detoxifying oxidants such as H_2O_2 or by decreasing intracellular labile iron to mitigate the effects of Fenton chemistry (61, 63, 77). H_2O_2 is removed enzymatically by catalase and alkyl hydroperoxide reductase (99), whereas superoxide is removed by SOD (63). Quantitative modeling in *E. coli* suggests that iron/sulfur-containing dehydratases and mononuclear iron enzymes are likely to be oxidized and repaired on a time scale of minutes under aerobic growth, even in cells having a normal complement of defensive enzymes (63, 104, 105). Any further increase in ROS requires the induction of defensive enzymes to an even higher titer (63). In *E. coli*, the induction of pathways for defense against ROS is controlled by the OxyR and SoxRS systems (63). Whereas OxyR senses H_2O_2 by disulfide bond formation, many other bacteria rely on a chemically distinct sensing mechanism involving metal-catalyzed oxidation of a regulator protein. The prototype for this class of regulators is the *Bacillus subtilis* PerR protein, a paralog of Fur (73).

The facile redox chemistry of ferrous iron requires that bacteria tightly regulate iron homeostasis and maintain an efficient mechanism to sense and respond to ROS such as H_2O_2 . Defenses against both iron limitation and ROS are important in the physiology of many pathogens. During infection, the host restricts bacterial growth by sequestration of essential metal ions, including iron, and innate immune cells kill bacteria using ROS and reactive nitrogen species (RNS) (36).

This review will focus on the role of Fur family metalloregulators in coordinating these two interacting adaptive responses. A special emphasis will be placed on the biochemistry and genetics of two Fur family proteins in the gram-positive soil bacterium *B. subtilis*, which will be designated as Fur_{Bs} and PerR_{Bs}. Comparison of the reactivity of these proteins and their orthologs with ROS and RNS reveals a wide range of sensitivity, and recent results have begun to shed light on how this reactivity may be tuned by changes in protein structure.

Fur Family Metalloregulators

The ferric uptake regulator (Fur) protein was first characterized in *E. coli* (Fur_{Ec}) where it functions as an iron-responsive repressor. Initial genetic studies revealed that *E. coli fur* null mutants produced very high levels of siderophores (51, 53). Subsequent biochemical characterization demonstrated that Fur_{Ec} is a dimeric, helix-turn-helix (HTH) containing DNA-binding protein that binds Fe^{2+} as corepressor (10, 11, 26). These observations, together with subsequent studies (89), led to a general model in which Fur_{Ec} directly senses Fe^{2+} levels in the cell to maintain iron homeostasis.

Fur family metalloregulators are ubiquitous in bacteria and are commonly identified through the conserved HHHXH₂CX₂C motif positioned at the hinge region between the metal-sensing C-terminus and the DNA-binding domain (40, 74). Although Fur proteins are most closely associated with iron homeostasis, it is now appreciated that the Fur family includes many proteins with

distinct metal selectivities. Variations in the metal-binding sites have enabled the evolution of Fur family members that are activated by a diverse set of metals. For example, the Fur family includes sensors of zinc (Zur), manganese (Mur), and nickel (Nur) (3, 53, 74). In addition, a subset of the iron-utilizing Fur family proteins functions physiologically as sensors of intracellular H_2O_2 (PerR) (73, 74).

Given the broad metal-sensing scope of these regulators, it is not surprising that bacteria may possess multiple Fur homologs. For example, *B. subtilis* has three Fur family paralogs: a Zn^{2+} -sensing Zur (45), an Fe^{2+} -sensing Fur, and a peroxide-sensing PerR (16, 91). Interestingly, *B. subtilis* does not sense Mn^{2+} via a Fur family protein, but rather through MntR, a member of the diphtheria toxin regulator (DtxR) family (59, 96). Furthermore, it was recently reported that *Bacillus licheniformis* not only has Fur and Zur but also contains three PerR paralogs (68).

Paralogous Fur family members may evolve distinct metal selectivity by changes in the chemical nature and geometry of the metal-coordinating ligands at the metal-sensing sites (74). Furthermore, each sensor must have an affinity appropriate for detecting fluctuations in the intracellular buffered pool of free metal ions, as reviewed in detail elsewhere (49, 112, 113). However, Fur family regulators can bind to noncognate metals either as agonists or antagonists. The binding of noncognate metals to metalloregulatory proteins (mismetallation) can have deleterious consequences for regulators, just as it can for enzymes. For example, in *E. coli* and related proteobacteria, high levels of Mn^{2+} are toxic and mutations that inactivate Fur often arise conferring resistance to these elevated manganese levels (52). This suggests that mismetallation of Fur with Mn^{2+} may inappropriately repress Fe^{2+} uptake. Similarly, in *B. subtilis*, a modest increase in Fur_{Bs} expression (in a *perR* null mutant) leads to mismetallation with Mn^{2+} and results in iron starvation (38, 78), and mismetallation of PerR_{Bs} with Zn^{2+} can lead to dysregulation of the PerR regulon resulting in heme intoxication (20).

Structural studies have provided insight into how Fur family proteins sense metals and, in the case of PerR, respond to oxidants (73, 74, 79, 95), as well as into how they interact with DNA (32, 47). In general, Fur family proteins are homodimeric, and DNA-binding proteins comprised two main domains. The amino-terminal domain contains HTH motif and binds to DNA. The C-terminal domain is required for metal sensing and mediates dimerization. This metal-sensing domain typically contains two to three metal-binding sites (Fig. 2A). The first (site 1) is a structural metal-binding site comprising four cysteine residues that interact with a single Zn^{2+} atom (Cys₄Zn). This site is required for protein folding and dimerization for many Fur family proteins (4, 25, 72, 111, 115). However, some Fur homologs lack a structural zinc site. Such is the case for *Pseudomonas aeruginosa* Fur (Fur_{Pa}), which only has one of the four conserved Cys residues, making the site functionally dispensable *in vivo*, and *Pseudomonas putida*, which lacks all four conserved Cys residues (76). The metal-sensing site in most Fur family proteins (site 2) is located in the C-terminal metal-binding domain and often bridges to the DNA-binding domain, thereby providing a mechanism for allosteric activation of DNA binding (40, 66). This is the key site for iron sensing by Fur and for peroxide sensing by PerR (73, 78). In some Fur proteins, including Fur_{Bs}, there is also a third metal-binding site (site 3) of poorly defined function (33, 78).

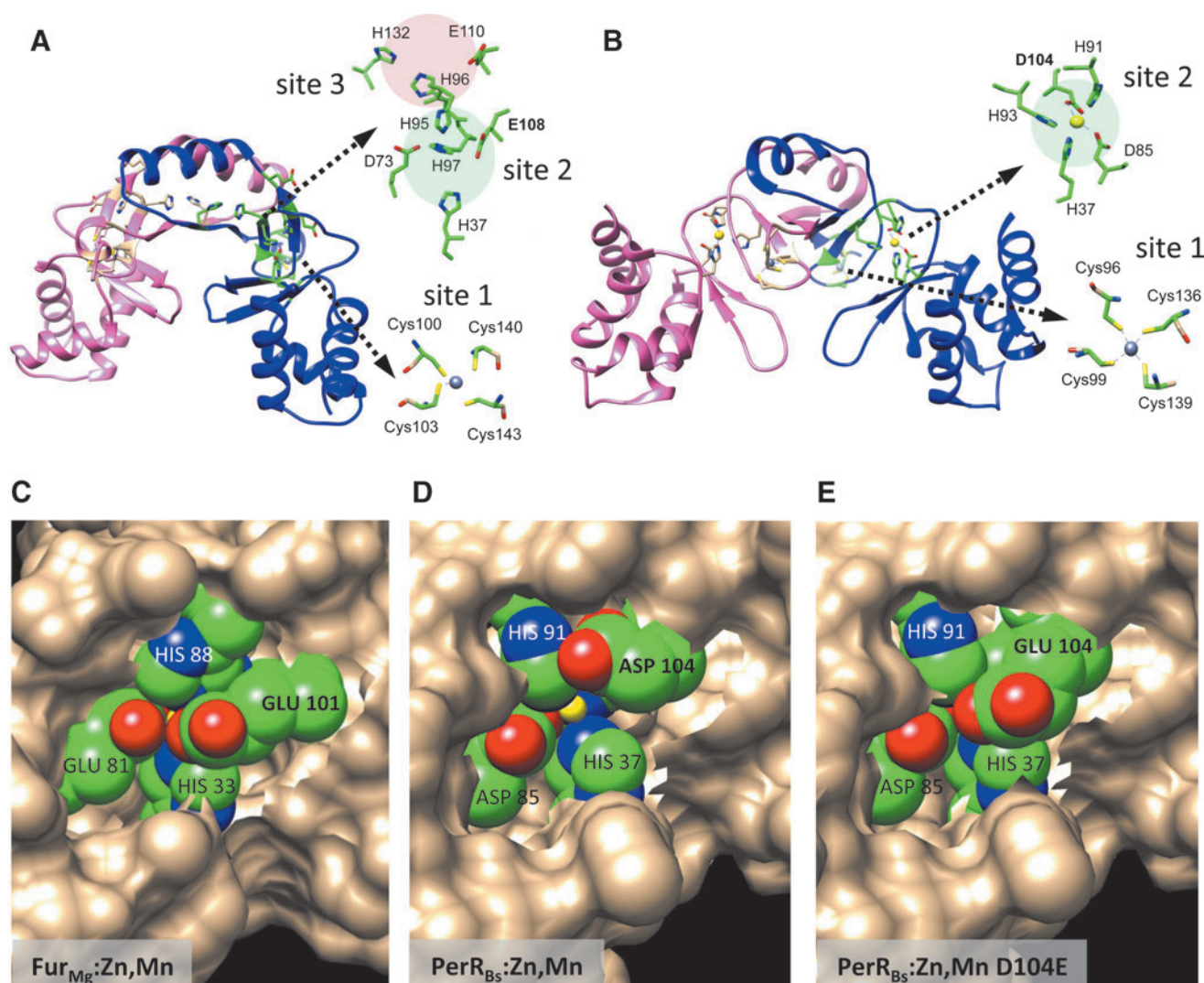


FIG. 2. Fur and PerR structures. (A) Fur_{BS} structural homology model based on the *Streptomyces coelicolor* Zur structure (PDB: 3mwm; sequence identity: 33.3%) (102). This Fur_{BS} model shows the predicted homodimer in its holo-form. Close-up views of its three conserved metal-binding sites are provided in stick form. Site 1 is a Cys-rich pocket that binds a structural Zn²⁺ atom to provide protein stability to the dimer. Sites 2 and 3 comprise the iron-sensing site (shown without metal atoms), where site 2 seems to play a primary role in triggering the conformational changes required for DNA binding [figure adapted from (78)]. (B) PerR_{BS} crystal structure bound with Mn²⁺. A close-up of the metal-sensing site (site 2) and the structural site (site 1) is depicted in stick form (PDB code: 3F8N). (C) Crystal structure of Fur_{Mg}:Zn,Mn (PDB code: 4RAZ) showing a close-up of binding site 1, which is analogous to site 2 in Fur_{BS} and in PerR_{BS}. Mn²⁺ is not easily accessible. (D) Crystal structure of PerR_{BS}:Zn,Mn (PDB code: 3F8N) showing a close-up of binding site 2. Mn²⁺ is easily accessible. (E) Crystal structure of PerR_{BS}:Zn,Mn showing a close-up of binding site 2 with conserved Asp104 residue mutated into a Glu. Mn²⁺ is completely occluded (Mn²⁺ = yellow; nitrogen atom = blue; oxygen atom = red; carbon atom = green). Asp, aspartate; Cys, cysteine; Fur, ferric uptake regulator; Glu, glutamate; His, histidine; Mn, manganese; Zn, zinc.

Iron Utilizing Fur Family Regulators in *B. subtilis*: Fur_{BS} and PerR_{BS}

B. subtilis has two Fur regulators that bind to iron: Fur_{BS} and PerR_{BS}. Although these two proteins are closely related and bind to the same divalent cation, their functions are quite distinct. This is evident from the set of genes they control (13, 37, 56, 91). Fur_{BS} controls a large regulon that comprises ~40 genes involved in iron homeostasis, consistent with its role as the primary sensor of iron limitation (13). PerR_{BS} is involved in controlling expression of genes that help combat H₂O₂ stress, including genes that encode iron efflux and

storage functions (21, 37, 48). Characterization of *B. subtilis* Fur and PerR illustrates an important feature of Fur family regulators: it is quite difficult to unambiguously assign function solely from inspection of protein sequence. Often, functional assignments can be best inferred by considering the set of genes regulated by each protein.

The Fur_{BS} regulon

The Fur_{BS} regulon can be divided into two main groups: iron acquisition genes and genes involved in the iron-sparing response. In the former, one subset is involved in siderophore

biosynthesis. Fur_{Bs} regulates the *dhb* operon, which encodes proteins involved in the synthesis of bacillibactin (BB) (13, 15, 46, 85). The second subset encodes ABC transporters involved in ferric siderophore uptake with specificity for BB and enterobactin (*feuABC*), ferrichrome (*fhuBCDG*), and schizokinen/arthrobactin (*yfiZY/yfhA*) (94). In addition, Fur_{Bs} regulates ferric citrate uptake (*yfmCDEF*) and elemental iron uptake (*efeOUB*) (Fig. 1) (13, 88, 94). Second, Fur controls expression of a small RNA (FsrA) and three RNA chaperones (FbpA, FbpB, and FbpC). When derepressed, FsrA targets specific genes that code for selected iron enzymes, inhibiting their translation and thus effectively carrying out the iron-sparing response, which efficiently prioritizes iron utilization in the cell (Fig. 1) (44, 103). This system is functionally analogous to the similar iron-sparing response of *E. coli* mediated by the Fur-controlled small RNA RyhB (81–83).

Fur recognizes genes within its regulon by binding to specific DNA operator sites (Fur boxes) located within the promoter region of its target genes. Originally, the Fur box was defined as a 19 bp inverted repeat (35), but subsequent studies demonstrated that a minimal-binding site is a heptameric inverted repeat motif (7-1-7): the 19 bp consensus corresponds to two, overlapping 7-1-7 motifs (12). The binding of Fur to these two overlapping binding sites has been visualized in crystals of *Magnetospirillum gryphiswaldense* Fur (Fur_{Mg}) metallated with Mn²⁺ and bound to a Fur box DNA sequence (32). Remarkably, all three Fur paralogs in *B. subtilis* recognize very similar inverted repeat motifs, but there is minimal overlap in their target genes (42). Specific recognition takes place, in part, through a conserved arginine (Arg) residue located at position 61 (R61) of the Fur_{Bs} DNA-binding domain (18). This residue recognizes the conserved guanine (G) and cytosine (C) bases located within the 7-1-7 motif (TGATAAT-N-ATTATCA), which are precisely the bases that distinguish Fur-binding sites from typical PerR-binding sites (42).

When *B. subtilis* cells experience iron depletion, the Fur regulon is derepressed. In the laboratory, iron depletion can be generated by treating cells with dipyrindyl, an intracellular iron chelator, thus affecting the labile iron pool (13). As free iron levels drop in the cell, iron is no longer bound to Fur. The resultant conformational change leads to dissociation from DNA, thus allowing RNA polymerase to bind to the previously occluded promoter regions and initiate transcription. These Fur-regulated genes are thereby regulated directly by Fur binding, as confirmed by DNaseI footprinting (13). Some Fur family regulators may retain regulatory activity in the apo-form and bind to sites distinct from those bound by the metallated holo-protein. This type of more complex regulation has been described for Fur from both *Campylobacter jejuni* (17) and *Helicobacter pylori* (1).

Regulon derepression can take place in one of two general ways. As iron levels fall, all members of the regulon may be derepressed simultaneously, or instead as a stepwise (graded) response where different subsets of genes are derepressed according to the level of stress the cell is experiencing. For example, the *B. subtilis* Zur regulon is derepressed in three distinct stages as bioavailable zinc levels decline (101). A similar effect is observed in the *Streptomyces coelicolor* Zur (102).

Fur regulons may also respond to gradual iron depletion by the sequential derepression of various adaptive mechanisms,

but the details are still emerging. The mechanisms underlying this sequential response are also not yet well understood, but may be related to variations in the architecture of the Fur-binding sites. For example, at some sites, Fur may function as a single dimeric protein and bind an isolated 7-1-7 motif (12). At other sites, Fur may bind to the classic 19 bp consensus as two, opposed dimers (binding on opposite faces of the DNA helix) (32, 47). Finally, Fur is known to have a propensity to bind to extended regions of DNA, and this cooperativity may be facilitated by protein/protein interactions (19, 29, 31, 41, 71). Differences in both the number and affinity of Fur-binding sites presumably help tune both the magnitude of the induction and the responsiveness of each target operon to the level of iron depletion.

Structural insights into Fe²⁺ sensing by Fur

The activity of Fur_{Bs} is regulated by its specific interaction with Fe²⁺. As noted above, Fur_{Bs} is representative of those Fur family members with a structural Zn²⁺ site and two additional metal-binding sites (Fig. 2A). Purification of Fur_{Bs} in the presence of metal chelators yields the stable, dimeric apo-protein containing only the structural Zn²⁺ ion (Fur_{Bs}:Zn) (78). Addition of Fe²⁺ (or other divalent cations) activates DNA binding with a stoichiometry of two Fe²⁺ atoms per monomer. The site 2 metal-sensing site is required for iron sensing. In addition, Fur_{Bs} contains a third site (site 3) near the dimerization interface. *In vitro*, Fe²⁺ first binds to site 3, followed by subsequent binding of the metal to site 2 (78). Although both site 2 and site 3 are required for DNA binding, site 2 is the major iron-sensing site; binding to site 3 has a relatively modest effect (~7-fold) on DNA-binding affinity compared to that achieved by binding to site 2 (~150-fold) (78).

The precise mechanism by which these two metal-sensing sites interact to trigger the conformational changes that activate DNA binding by Fur remains unclear (74, 78). It is possible that the sequential binding of Fe²⁺ to site 3 and then site 2 helps to fine-tune the expression of genes within the Fur regulon, or to help Fur_{Bs} avoid inappropriate activation by mismetallation. For example, sensing of Mn²⁺ by the DtxR family protein MntR involves the sequential loading of Mn²⁺ into two neighboring sites in each monomer, which is thought to provide a mechanism to increase the selectivity of metal responsiveness (86). Alternatively, site 3 may play a largely structural role in stabilizing the dimeric repressor. Indeed, it has not even been established whether or not Fe²⁺ is the metal that occupies site 3 *in vivo*.

Under normal physiological conditions, Fur binds specifically to Fe²⁺, and other divalent metal ions do not elicit repression of the Fur regulon. The concentration of free iron required to trigger DNA binding by Fur_{Bs} is defined by the K_d for Fe²⁺ binding (~1 μM) *in vitro*, which thereby provides an estimate of the buffered concentration of free Fe²⁺ in the cell. A nearly identical affinity (~1.2 μM) has been measured for Fur_{Ec} (89).

However, *in vitro* studies reveal that Fur_{Bs} can also be activated by Mn²⁺, although with a lower binding affinity (K_d of ~24 μM) (78). Typically, the cellular concentration of free Mn²⁺ is maintained at a level too low to mismetallate Fur_{Bs}. Mn²⁺ levels are regulated by MntR, which both represses uptake and activates efflux of Mn²⁺ and has an Mn²⁺

affinity ($K_d \sim 6 \mu M$) several fold higher than that of Fur_{BS} ($K_d \sim 24 \mu M$). Interestingly, under conditions where Fur_{BS} is overexpressed (as is the case in a *perR* null strain), Fur_{BS} is now constitutively activated by physiological levels of intracellular Mn²⁺ (78). In addition, Fur_{BS} has also been shown to exhibit some cross talk with Cd²⁺ or Zn²⁺ in the presence of sufficiently high concentrations of these metals (90), but it is unknown if this is mediated by direct binding of these cations to Fur_{BS} or indirectly. The high selectivity of Fur_{BS} for Fe²⁺ *in vivo* is remarkable in light of the many genetic studies suggesting that mismetallation of Fur by Mn²⁺ leads to dysregulation of the Fur regulon in many proteobacteria, including *E. coli* and *Salmonella enterica* (52, 60).

The PerR regulon

PerR_{BS} was originally identified as a Fur family protein required for the adaptation of *B. subtilis* to H₂O₂ stress (16, 119). The PerR_{BS} regulon comprised two groups of genes: (i) those involved in the direct detoxification of ROS and (ii) those that contribute to iron homeostasis. The former group consists of *katA* (vegetative catalase) and *ahpCF* (alkyl hydroperoxide reductase), both of which encode enzymes involved in the direct detoxification of hydrogen peroxide (H₂O₂) (16, 22, 37, 63). The heme biosynthesis operon (*hemAXCDBL*) also falls under this category, since induction of heme biosynthesis supports the activity of the abundantly expressed, heme-dependent catalase KatA (38) (Fig. 1). The second group of PerR-regulated genes encodes a Dps-like miniferritin involved in iron sequestration (MrgA) (21, 23) and a P_{1B4}-type ATPase involved in ferrous iron efflux (PfeT) (Fig. 1) (48). Finally, PerR also controls *fur* and autoregulates its own expression (41, 54). The observation that PerR_{BS} specifically regulates H₂O₂ detoxification and iron sequestration (MrgA), efflux (PfeT), and import (indirectly through Fur) is consistent with a primary role in minimizing the damage elicited by iron-catalyzed Fenton chemistry.

Early genetic studies revealed that *B. subtilis* peroxide stress genes were regulated by both metal ions and by H₂O₂, and this regulation required the same cis-acting operator site and the same trans-acting factor (22). This coordinate regulation is explained by the role of PerR_{BS} as a metal-dependent repressor (strongly activated by either Fe²⁺ or Mn²⁺). Like other Fur family proteins, PerR_{BS} contains a structural Zn²⁺ (site 1) and metal-sensing site 2 (Fig. 2B). PerR_{BS} is highly sensitive to H₂O₂ inactivation when in its Fe²⁺-cofactored form (designated PerR:Zn,Fe). In response to H₂O₂ stress, the *mrgA*, *katA*, and *pfeT* genes are all highly induced (55) (Fig. 3). The *ahpC* and *hemA* promoters are also modestly induced by H₂O₂, and PerR_{BS} also exhibits a weak (twofold to threefold) repression of *fur* and *perR* (43) (Fig. 3).

Operator sites for PerR-regulated genes (Per boxes) are very similar to those for Fur (42). They both align as 7-1-7 motifs and have a high sequence similarity with the exception of thymine (T) and adenine (A) at the -6 and +6 positions, respectively, of the two heptamers (TTATAAT-ATTATAA). These two bases are typically a G and a C in Fur boxes. These conserved bases are recognized by an asparagine (Asn) residue in position 61 of PerR_{BS} (18). A similar inverted repeat pattern is also observed in Zur boxes, additionally requiring a three base pair extension on both sides of the minimal

operator (42). Although these operator sites are very similar, there is little if any overlap of regulators across operator sites.

Structural insights into H₂O₂ sensing by PerR

Much like Fur_{BS}, PerR_{BS} is a dimeric DNA-binding protein with two distinct functional domains: a DNA-binding domain and a sensing domain. PerR_{BS} binds two metal ions per monomer (72, 73, 78, 79, 108), instead of three as noted for Fur_{BS} (78) (Fig. 2A, B). The first is a structural Zn²⁺ atom necessary for structural integrity and protein dimerization. This Zn²⁺ is bound tightly to PerR_{BS} in a cysteine pocket (Cys₄:Zn) and remains intact even with high concentrations of peroxides. Indeed, this Zn²⁺ is often retained in the protein structure even during denaturing sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) (72). The binding of a second metal to the metal-sensing site 2 leads to activation of DNA binding. In cells, PerR can be metallated by either Fe²⁺ or Mn²⁺ simply by altering the availability of metals in the growth medium (43, 56). In most iron-rich media, PerR_{BS} is bound with Fe²⁺ and, in this state, is responsive to H₂O₂ (56). However, addition of excess Mn²⁺ can shift PerR into the Mn²⁺-activated form (PerR:Zn,Mn) and in this state, peroxide stress genes are tightly repressed (Fig. 3).

Typically, peroxide sensing is mediated by redox active cysteines, in sensors such as OxyR in *E. coli* (116) and OhrR in *B. subtilis* (75, 106). These are metal-independent peroxide sensors that detect ROS *via* the oxidation and subsequent formation of reversible disulfide bonds (8, 14, 34, 107). This contrasts with PerR_{BS}, which senses H₂O₂ by metal-catalyzed oxidation through site 2 (73). Binding of H₂O₂ to Fe²⁺ in PerR:Zn,Fe is proposed to generate a localized hydroxyl radical that modifies one of two His residues (H37 or H91) in site 2 to 2-oxo-histidine (Fig. 4) (73, 98, 109). Recently, an alternative mechanism has also been suggested in which the initial bound H₂O₂ undergoes heterolytic cleavage releasing water and generating an Fe(IV) oxo intermediate (100). His oxidation may also occur on direct reaction of PerR:Zn,Fe with O₂, and the presence of O₂ also stimulates the modification by H₂O₂ (Fig. 4) (100). Interestingly, these two oxidizing agents appear to use distinct modification pathways: reaction with H₂O₂ targets H37 and H91 at about equal rates, whereas reaction with O₂ selectively leads to H37 modification (100) (Fig. 4).

Regardless of the precise pathway, oxidized PerR loses its iron cofactor and the resulting conformational changes trigger its release from DNA and exposes specific signature residues targeted by the LonA protease (Fig. 3) (2). Thus, after regulon derepression occurs, oxidized PerR_{BS} is degraded thereby preventing the accumulation of nonfunctional protein (2). Oxidation of PerR_{BS} also leads to derepression of the autoregulated *perR* gene, which presumably helps re-establish repression once the newly synthesized PerR:Zn,Fe protein is no longer consumed by reaction with H₂O₂. Transcriptomic studies demonstrate that derepression of the PerR regulon in response to H₂O₂ is transient and repression is rapidly re-established (55). This is likely important since constitutive derepression of the PerR regulon, such as seen in a *perR* null mutant, imposes a very high iron demand on the cell due to the high levels of catalase that are produced (38, 78). This iron deficiency imposed in a *perR* null mutant is also observed in *B. licheniformis*, where overexpression of

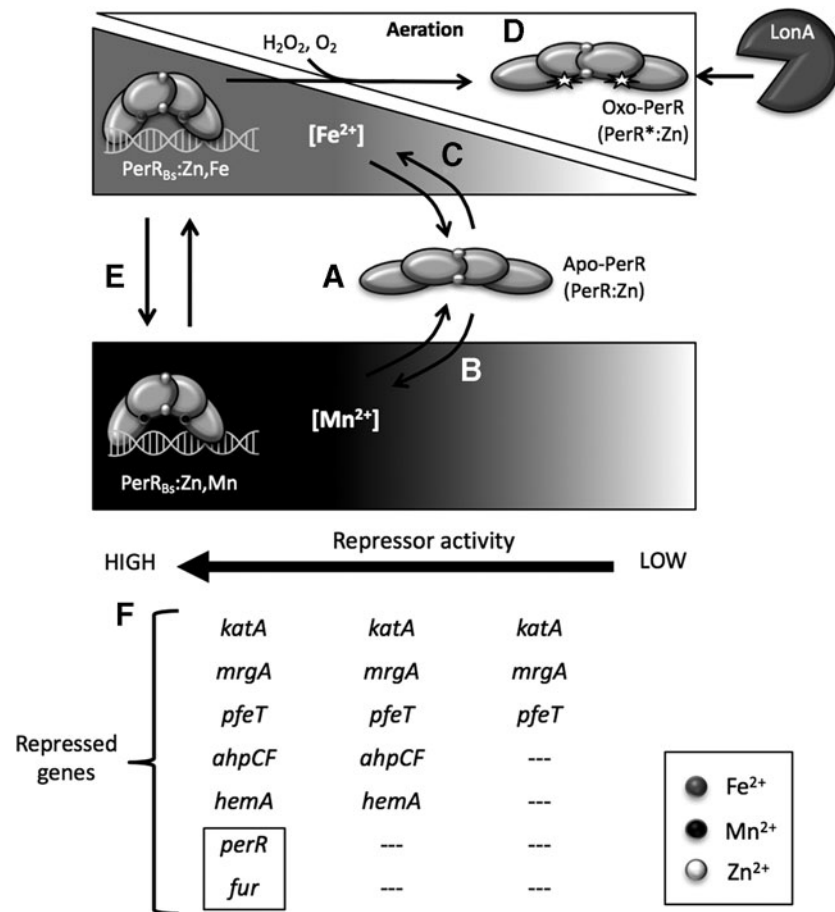


FIG. 3. Mechanism of gene regulation by PerR_{Bs}. (A) Apo-PerR (PerR:Zn) lacks a metal cofactor at its metal-sensing site. This causes PerR to adopt a conformation that prevents it from binding to its DNA operator sites. As a result, no gene repression takes place. (B) As Mn²⁺ concentrations increase, apo-PerR becomes metallated, triggering a conformational change in PerR:Zn,Mn allowing it to bind its specific operator sites and repress PerR-regulated genes. Note that PerR:Zn,Mn is insensitive to oxidizing agents. (C) As Fe²⁺ concentrations increase, apo-PerR becomes metallated by iron. PerR:Zn,Fe binds to PerR operator sites and represses genes in the PerR regulon. However, during aerobic growth or in the presence of ROS, the ability of PerR:Zn,Fe to act as a repressor is limited by protein oxidation. (D) Oxidation of PerR exposes a conserved signature residue sequence recognized by LonA. Thus, oxo-PerR is targeted for degradation by the protease, preventing accumulation of inactive protein. (E) Metallated PerR can reversibly bind to Mn²⁺ or Fe²⁺ as metal concentrations in the cell vary. (F) Overview of the ability of PerR to repress various genes as a function of metal availability. In the presence of iron, under aerobic conditions, inactivation of PerR prevents accumulation of high levels of active repressor. As a result, metallated PerR:Zn,Fe is most efficient at repression of genes postulated to have high-affinity operator sites (*katA*, *mrgA*, *pfeT*). PerR:Zn,Mn, which is not susceptible to oxidation, can accumulate to a high effective level and strongly represses genes the entire PerR regulon, including those genes postulated to have the lowest affinity operator sites (*perR*, *fur*) (as highlighted by box). ROS, reactive oxygen species.

fur, rather than *katA*, is the main reason why cells cannot grow in media not supplemented with iron (70).

Metal-dependent changes in PerR_{Bs} repressor activity

In addition to Fe²⁺, PerR_{Bs} also binds Mn²⁺ to generate PerR:Zn,Mn. This form of PerR also represses the PerR regulon but is peroxide insensitive and fails to derepress the PerR regulon in response to H₂O₂ (43, 73). The reason why PerR_{Bs} binds to both Fe²⁺ and Mn²⁺ is not well understood: in *B. subtilis* the PerR regulon does not control any manganese-related genes. In contrast, *E. coli* OxyR, which also senses H₂O₂, directly activates the expression of the NRAMP family manganese importer MntH in response to H₂O₂ stress (7, 37,

80). This is not the case in *B. subtilis*, where manganese homeostasis is maintained independently of PerR_{Bs}, via MntR (59). The repression of the PerR_{Bs} regulon by Mn²⁺ may have resulted as a corollary of comparatively high affinity of PerR for Fe²⁺: PerR_{Bs} binds Fe²⁺ tighter than Fur_{Bs} (79), and this may have also led to a high Mn²⁺ affinity. This high Fe²⁺ affinity ensures that the peroxide stress response is not induced by mild iron depletion, which would be maladaptive and impose a high iron demand through the increased synthesis of heme and catalase and the expression of proteins mediating iron sequestration (MrgA) and efflux (PfeT). Hence, PerR may have sacrificed metal selectivity to help increase its specificity to respond to H₂O₂ stress rather than fluctuations in metal availability.

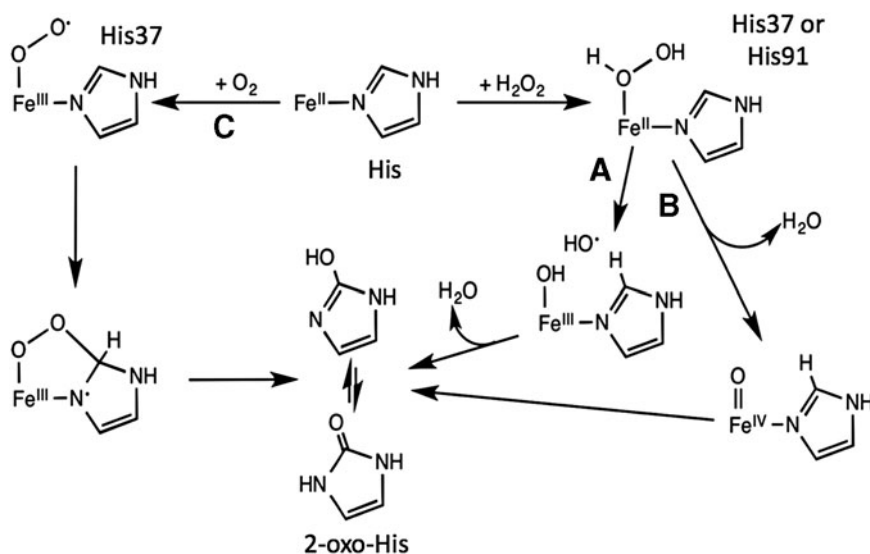


FIG. 4. Metal-catalyzed oxidation of PerR. Formation of 2-oxo-histidine of PerR_{Bs}:Zn,Fe site 2 via three putative pathways. His37 or His91 can both become oxidized when exposed to H₂O₂. This can lead to either (A) the generation of a hydroxyl radical via oxidation of Fe²⁺ into Fe³⁺ as a result of the homolytic cleavage of the O-O of the peroxido intermediate or (B) the production of a high valent Fe⁴⁺ ion (especially under pH ~7.0) caused by the heterolytic cleavage of that same bond. Conversely, (C) in the presence of O₂, His37 is specifically targeted, resulting in a superoxo-Fe³⁺ intermediate. Subsequent bond formation and O-O bond cleavage of the end derivative leads to the final production of 2-oxo-His [figure adapted from (100)].

The binding of either Fe²⁺ or Mn²⁺ may additionally allow PerR to function as a ratiometric sensor of the relative iron and manganese levels. For example, when the level of Mn²⁺ is much higher than Fe²⁺ in the cell, the threat posed by H₂O₂ may be significantly reduced, thereby justifying a reduced induction of PerR regulon genes. Furthermore, there is suggestive evidence that the PerR:Zn,Fe and PerR:Zn,Mn forms of the repressor may differentially affect some genes (43) (Fig. 3). For example, in resuspension experiments, either Mn²⁺ or Fe²⁺ enabled repression of some PerR-regulated genes (*kata*, *ahpCF*, *mrgA*), whereas only Mn²⁺ elicited repression at the *pfeT*, *per*, and *fur* promoters. The basis for this Mn²⁺ selectivity is presently unknown, but two models have been suggested. First, PerR:Zn,Fe and PerR:Zn,Mn may be qualitatively different: they may have slightly different conformations that affect their DNA-binding selectivity. Second, the difference may be primarily quantitative: the much more stable PerR:Zn,Mn form may accumulate in cells to a higher active concentration allowing interaction with weaker operators within the PerR regulon (Fig. 3). Regardless of the mechanism, these observations are relevant when we consider below PerR orthologs that were reported to be specific for Mn²⁺ as a corepressor.

Structural differences that tune H₂O₂ sensitivity (it is the little things that count)

Despite the overall structural similarity between Fur_{Bs} and PerR_{Bs}, they differ greatly in their sensitivity to H₂O₂. PerR_{Bs} is highly sensitive to H₂O₂ with a second-order rate constant for protein oxidation of ~10⁵ M⁻¹ s⁻¹ when associated with iron (73). This is comparable to the reported sensitivity of OxyR, which responds to submicromolar levels of intracellular H₂O₂ (9). As a result, isolation of unoxidized PerR:Zn is

technically challenging and is best achieved by purification from cells grown with high Mn²⁺ (or in medium amended with iron chelators) to prevent *in vivo* oxidation, lysis, and purification in the presence of EDTA to remove any Fe²⁺ from the protein, and exclusion of thiol reducing agents (which can facilitate reduction of Fe³⁺ to Fe²⁺) (73, 100). The high sensitivity of PerR_{Bs} to H₂O₂ inactivation is appropriate for its role in regulating detoxification functions.

In contrast with PerR, Fur proteins are generally less sensitive to H₂O₂ inactivation, which is likely adaptive since the derepression of iron uptake functions in response to elevated H₂O₂ levels would likely be deleterious to the cell. Compared to PerR_{Bs}, Fur_{Bs} is relatively easy to purify in an active state, although care is needed to prevent the adventitious binding of metals to the sensing site(s) (15). As discussed in more detail below, Fur proteins from a variety of organisms can be activated *in vitro* by addition of Fe²⁺ as a corepressor, even in aerobic buffers.

Recent results have begun to provide insights into how PerR_{Bs} and Fur_{Bs} display such different sensitivities to H₂O₂. Both PerR_{Bs} and Fur_{Bs} have a histidine-rich binding pocket (site 2) that coordinates Fe²⁺ (15, 54, 74). However, an aspartate (Asp) residue in PerR_{Bs} (position 104) replaces a glutamate (Glu) in Fur_{Bs} (position 108) as the Fe²⁺ ligand (95). Mutational studies demonstrate that the PerR_{Bs} D104E and Fur_{Bs} E108D mutants still bind Mn²⁺ with near-normal affinities. However, the sensitivity of each protein to metal-catalyzed oxidation of the neighboring histidine ligands (H37 and H91 in PerR and the equivalent residues in Fur) was significantly reduced. This was assessed using electrospray ionization mass spectrometry analysis to monitor His oxidation in proteins overexpressed and purified from aerobically growing *E. coli*, which generally maintains steady-state levels of H₂O₂ in the range of 50 nM. Under these conditions, ~63% of the recovered PerR was

oxidized, with a much lower value noted for Fur (4%). Significantly, oxidation of the PerR_{Bs} D104E mutant was substantially reduced (to 3%) whereas that for Fur_{Bs} E108D was somewhat increased (to 14%) (95). These results were corroborated by *in vitro* analyses of protein oxidation by H₂O₂.

These mutational studies support a model in which the H₂O₂ reactivity of the bound Fe²⁺ can be quantitatively modulated by single amino acid changes in site 2 that affect coordination geometry. Structural studies of PerR:Zn,Mn revealed a penta-coordinate site 2 with three His and two Asp residues (32, 66). Assuming Fe²⁺ binds similarly to Mn²⁺, this leaves a sixth coordination site available for interaction with incoming H₂O₂. Structural modeling supports the notion that substitution of the PerR Asp104 ligand with the longer Glu side chain allows formation of a weak bidentate interaction between Glu and Fe²⁺ that occludes access of H₂O₂ and thereby minimizes protein oxidation (95) (Fig. 2C–E). The resultant Fe²⁺-binding site can be considered to have a “5 + 1” coordination mode as proposed previously for Fur based on Mössbauer and X-ray absorption spectroscopy (65). Recent structural studies of *M. gryphiswaldense* Fur indicate that Mn²⁺ (bound as an iron surrogate) is indeed hexacoordinate in this protein, with three His and two Glu ligands (32).

Collectively, these results support a simple model in which PerR proteins bind Fe²⁺ with five ligands and one open coordination site that can interact with H₂O₂, whereas Fur proteins use six ligands and the interaction of H₂O₂ with Fe²⁺ is sterically impeded. It would be revealing to determine if these same types of steric access effects influence the reactivity of bound Fe²⁺ with other molecules. In addition to H₂O₂, PerR can also be inactivated by O₂ (100) and nitric oxide (NO) inactivates Fur_{Ec} by direct nitrosylation of the bound Fe²⁺ (27, 28). Transcriptomic studies indicate that NO, as well as sodium nitroprusside, also inactivates both PerR_{Bs} and Fur_{Bs} under both aerobic and anaerobic conditions (92). Finally, it has been shown *in vitro* that Fe²⁺ bound to Fur can be oxidized by K₃Fe(CN)₆ (89). It would be interesting to test the ability of these various redox agents to interact with PerR_{Bs} and Fur_{Bs} and the corresponding mutants affecting the Fe²⁺ ligands. However, it is likely that it will ultimately be necessary to account for second coordination sphere effects and allosteric coupling to fully understand metal selectivity and peroxide reactivity, as noted in biophysical studies of other metalloregulators (49).

PerR and Fur Orthologs Across Species: Key Directions for Further Work

The revelation that varying a single amino acid in the Fe²⁺ coordination sphere of Fur/PerR type proteins can modulate H₂O₂ sensitivity (Fig. 2) is an important first step in understanding how these regulators have evolved distinct physiological roles. As evident from the literature, it is likely that there is a wide variation in H₂O₂ sensitivity among Fur and PerR orthologs in other organisms, and we are yet to tackle the complex problem of understanding how this variation has emerged. Certainly, variations in protein structure and the metal coordination environment are important factors, but the most relevant data relate to H₂O₂ sensitivity *in vivo*, and variations in the cellular environment are also likely important in interpreting the observed differences. As noted above,

it is clear from numerous studies that Fur and PerR proteins can function with either Fe²⁺ or Mn²⁺ as corepressors. However, these two forms cannot be considered as equivalent. This is important when considering *in vitro* studies that often use Mn²⁺ as a cofactor to activate DNA binding, even for Fur proteins that function physiologically to sense Fe²⁺. With these caveats in mind, we here review recent insights into the effects of H₂O₂ and other oxidants on Fur and PerR function across a variety of bacteria, and identify some key questions to guide future research.

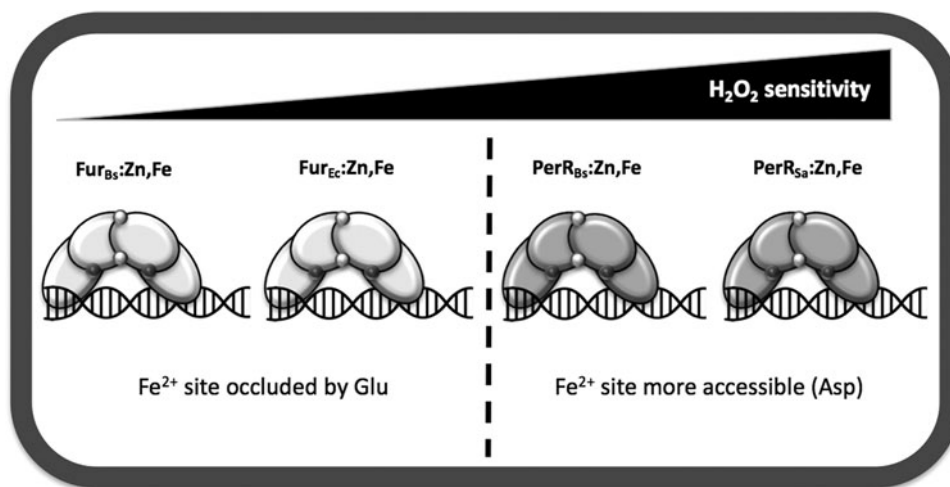
PerR orthologs with varying susceptibility to oxidation

PerR orthologs have been identified in a wide range of bacteria and seem to exhibit significant variation in their sensitivity to oxidation. However, comparative sensitivities are difficult to derive, given the differences in experimental parameters cited across the literature, including differences in media and treatment protocols for each organism tested. For example, the facultative anaerobe *Staphylococcus aureus* regulates peroxide stress using a PerR ortholog designated PerR_{Sa} (67% identical to PerR_{Bs}). Initial reports indicated that PerR_{Sa} repressed its target genes (*e.g.*, *katA*, *ahpCF*, *mrgA*) only in the presence of Mn²⁺ (24, 58, 67). Indeed, in Fe²⁺-amended medium, the expression of *katA* was as high as in a *perR* null mutant. Although these studies led to the suggestion that PerR_{Sa} is functionally distinct, and is specialized to use Mn²⁺ as corepressor, this seems unlikely since this form (PerR:Zn,Mn) would not be able to respond to peroxide using the mechanism established for PerR_{Bs} (Fig. 4).

An alternative explanation is that under the aerobic growth conditions used, PerR_{Sa} is oxidized by endogenously generated H₂O₂ and only the Mn²⁺ form is able to accumulate to levels needed to effect repression. Support for this model is provided by recent *in vivo* and *in vitro* studies that allow a direct comparison of PerR_{Sa} and PerR_{Bs} (67). These studies reveal that the apparent lack of Fe²⁺-dependent repressor activity for PerR_{Sa} reflects a hypersensitivity to protein oxidation under aerobic culture conditions: Fe²⁺ is an effective corepressor under microaerobic conditions and the cell is poised to respond to increased oxygen availability and the resultant production of ROS (67). Biochemical studies also indicate that PerR_{Sa} maintains its ability to bind to both Fe²⁺ and Mn²⁺, and that PerR_{Sa}:Zn,Fe is more sensitive to H₂O₂ than either PerR_{Bs} or *B. licheniformis* PerR (PerR_{Bl}) (67, 69). This increased sensitivity likely confers this facultative anaerobic pathogen with the necessary fine-tuning to detect lower levels of peroxides.

PerR orthologs from other facultative aerobes and strict anaerobes may have similarly increased sensitivity to oxidation and may thereby function for survival under aerobic conditions (aerotolerance). Such is the case for *Clostridium acetobutylicum* PerR (PerR_{Ca}), which controls genes important for survival in the presence of H₂O₂ or when exposed to air (57). The sulfate reducing, obligate anaerobe *Desulfovibrio vulgaris* Hildenborough also may contain a highly sensitive PerR (114). In this organism, cells exposed to air for 30 min were more tolerant to subsequent challenge with H₂O₂. Similarly, PerR in the microaerophilic food pathogen *C. jejuni* is important for adaptation to aerobic conditions (50).

FIG. 5. Different iron-binding Fur family regulators display different sensitivities to H₂O₂. Variation in H₂O₂ sensitivity among Fur family metalloregulators. The key difference seems to lie in the structural variations between Fur and PerR at the metal-sensing site. However, other differences also likely affect H₂O₂ sensitivity. PerR_{Sa}:Zn,Fe is more sensitive to oxidation by H₂O₂ than PerR_{Bs}:Zn,Fe, and Fur_{Ec} seems to be more sensitive to peroxides than Fur_{Bs}, although the basis for these differences has not been completely defined.



It is presently unclear whether PerR functions during the transition from anaerobic to aerobic conditions by sensing endogenously produced H₂O₂ or whether O₂ itself suffices (100). Indeed, recent results indicate that O₂ can modify PerR_{Bs} directly (100). Since this modification proceeds by a chemically distinct pathway (Fig. 4) and at least with PerR_{Bs} leads to a notable increase in the ratio of His37 to His91 oxidation (100), it may be possible to determine whether O₂ is the true signal for PerR orthologs that function to sense the transition from anaerobic to aerobic growth conditions.

Fur orthologs with varying sensitivity to oxidation

Fur orthologs have also been identified from many different bacteria, and subject to the same caveats noted above, they seem to display a wide variation in their sensitivity to oxidation. Insights into the relative susceptibility to oxidation can be obtained by comparisons of the susceptibility of Fur proteins to inactivation *in vitro* and *in vivo*, although in both cases detailed side-by-side comparisons have generally not been done, and few studies have directly addressed the issue of sensitivity to ROS.

The vast majority of biochemical studies of Fur proteins have as their major goal the demonstration that Fur acts directly at a specific target promoter region, and for this purpose it is common to metallate Fur with the more redox stable Mn²⁺ rather than the more relevant Fe²⁺ cofactor. Nevertheless, there are several reports of activation of DNA binding by Fe²⁺ *in vitro* (1, 15). This contrasts with PerR_{Bs}, which is rapidly inactivated if Fe²⁺ is added to air-saturated buffer (73). Indeed, the original demonstration that Fur_{Ec} functions as a direct sensor of Fe²⁺ was obtained by addition of Fe²⁺ to a coupled *in vitro* transcription/translation reaction (10) and subsequent DNA footprinting studies demonstrating activation of Fur binding by Fe²⁺ (30). Similarly, *H. pylori* Fur was active *in vitro* when reconstituted with Fe²⁺ as monitored by DNase I footprinting (1, 31). Since DNA-binding activity was monitored aerobically in these studies, this suggests that the iron metallated form of Fur proteins is often relatively stable, even in air-saturated buffers. In one of the few side-by-side comparisons to date, incubation of anaerobically reconstituted Fur_{Bs} with bound Fe²⁺ with a single molar equivalent (50 μM) of H₂O₂ led to a modest level of protein

oxidation (23%), but notably less than that seen for PerR_{Bs} under identical conditions (66%) (95).

We can also glean insights into the reactivity of Fur proteins to ROS from the large number of transcriptome and proteome studies of cellular responses to redox stress. For example, treatment of *B. subtilis* with either a low (8 μM) or high (58 μM) dose of H₂O₂ strongly induces the PerR regulon, but had only a modest effect, and only at the higher concentration, on Fur-repressed genes (55). However, a second study did find substantial derepression of the Fur regulon in cells treated with 58 μM H₂O₂ as well as by superoxide stress (93). Early studies also indicated that the *E. coli* Fur regulon was relatively insensitive to H₂O₂. Treatment of cells growing in rich medium with 1 mM H₂O₂ for 10 min strongly induced the OxyR-mediated stress response, but did not derepress the Fur regulon (118). In contrast with this result, an *E. coli* strain (*hpx*⁻) lacking all three major peroxide detoxification enzymes (*katG*, *katE*, and *ahpCF*) was found to derepress the Fur regulon in minimal medium. In this strain, endogenously generated H₂O₂ accumulates to levels between 500 nM and 1 μM, suggesting that this level is sufficient for oxidative inactivation of Fur (110). The authors further show that this derepression is not observed in rich medium due to the OxyR-dependent upregulation of Fur synthesis, which increases Fur levels from ~5000 to 10,000 molecules per cell (117). The nature of the oxidative inactivation of Fur_{Ec} is unclear and could result from oxidation and loss of the bound Fe²⁺ corepressor, with or without accompanying protein oxidation. It has been noted, however, that oxidation of Fur_{Ec} bound with Fe²⁺ to the Fe³⁺ bound state did not appreciably decrease DNA binding (89), supportive of a model in which metal-catalyzed oxidation is required for protein inactivation.

Conclusions and Perspectives

Iron sensing and oxidative stress are intricately intertwined. The major physiological effects from ROS often involve the inactivation of iron-dependent enzymes. We have here reviewed how *B. subtilis* used two functionally distinct members of the Fur family of metalloregulators, Fur_{Bs} and PerR_{Bs}, to coordinate the adaptive responses to iron limitation and peroxide stress, respectively. These proteins provide an attractive model system to begin to understand how

protein structural changes can modulate both the metal selectivity and the sensitivity to H₂O₂ and other oxidants in this family of regulators. Fur and PerR proteins are widespread among bacteria, yet available evidence suggests that they vary markedly in their sensitivity to redox active compounds. These proteins seem to occupy a continuum of reactivity from highly sensitive (PerR proteins from facultative aerobes and anaerobes) to relatively nonreactive (some Fur proteins) (Fig. 5). Unfortunately, controlled side-by-side comparisons of redox reactivity are rare. PerR proteins are typically inactivated by a wide variety of redox stresses (O₂, H₂O₂, HOCl, NO), and a subset of these agents, although sometimes only at much higher levels, may also affect Fur activity. Recent studies are beginning to shed light on some of the key differences in the metal-sensing sites that modulate redox sensitivity, but it is clear that much work remains to be done.

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Abbreviations Used

A	= adenine
AhpC	= alkyl hydroperoxide reductase
Arg, R	= arginine
Asn, N	= asparagine
Asp, D	= aspartate
BB	= bacillibactin
Cys	= cysteine
C	= cytosine
DtxR	= diphtheria toxin regulator
EDTA	= ethylenediaminetetraacetic acid
Fe	= iron
Fur	= ferric uptake regulator
Glu, E	= glutamate
G	= guanine
HTH	= helix-turn-helix
HOC1	= hypochlorous acid
His, H	= histidine
H ₂ O ₂	= hydrogen peroxide
KatA	= catalase
Mn	= manganese
NO	= nitric oxide
O ₂	= oxygen
RNS	= reactive nitrogen species
ROS	= reactive oxygen species
SDS-PAGE	= sodium dodecyl sulfate/polyacrylamide gel electrophoresis
SOD	= superoxide dismutase
T	= thymine
Zn	= zinc