Peroxide Stress Elicits Adaptive Changes in Bacterial Metal Ion Homeostasis

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Abstract

Exposure to hydrogen peroxide (H_2O_2) and other reactive oxygen species is a universal feature of life in an aerobic environment. Bacteria express enzymes to detoxify H_2O_2 and to repair the resulting damage, and their synthesis is typically regulated by redox-sensing transcription factors. The best characterized bacterial peroxide-sensors are *Escherichia coli* OxyR and *Bacillus subtilis* PerR. Analysis of their regulons has revealed that, in addition to inducible detoxification enzymes, adaptation to H_2O_2 is mediated by modifications of metal ion homeostasis. Analogous adaptations appear to be present in other bacteria as here reviewed for *Deinococcus radiodurans*, *Neisseria gonorrhoeae*, *Streptococcus pyogenes*, and *Bradyrhizobium japonicum*. As a general theme, peroxide stress elicits changes in cytosolic metal distribution with the net effect of reducing the damage caused by reactive ferrous iron. Iron levels are reduced by repression of uptake, sequestration in storage proteins, and incorporation into metalloenzymes. In addition, peroxide-inducible transporters elevate cytosolic levels of Mn(II) and/or Zn(II) that can displace ferrous iron from sensitive targets. Although bacteria differ significantly in the detailed mechanisms employed to modulate cytosolic metal levels, a high Mn:Fe ratio has emerged as one key correlate of reactive oxygen species resistance. *Antioxid. Redox Signal.* 15, 175–189.

Opportunities and Challenges Afforded by Molecular Oxygen

MOLECULAR OXYGEN is a defining feature of the Earth's atmosphere due to both its abundance (21% by volume at sea level) and its unique chemical reactivity. As a ground state radical, carrying two unpaired electrons, O_2 reacts readily with a variety of one-electron reductants. This ability has been harnessed by both eukaryotes and many prokaryotes, and aerobic respiration is arguably the dominant form of cellular energy generation. Growth in the presence of oxygen also poses challenges for cells since partially reduced oxygen species (reactive oxygen species [ROS]) can damage many cellular constituents leading to protein and membrane damage, mutations, and ultimately cell death (39). Thus, the evolution of an oxygen-containing atmosphere, as a result of oxygenic photosynthesis by early microbes, provided both new opportunities (for energy generation) and chemical challenges for evolving life forms (40).

Hydrogen peroxide (H_2O_2) is among the most stable ROS and consequently can accumulate to significant levels both within cells and in the environment. H_2O_2 forms within cells by the auto-oxidation of flavins associated with metabolic enzymes and, as a small uncharged molecule, can also enter by diffusion from the environment (50). Auto-oxidation re-actions in air-saturated environments can lead to micromolar levels of H_2O_2 and many organisms (animals, plants, and some bacteria) produce even higher levels as an antibacterial weapon (88).

Bacterial cells routinely express multiple enzymes dedicated to the detoxification of H_2O_2 and other ROS. The ubiquity of catalases, peroxidases, and superoxide dismutases (SODs)/reductases provides compelling evidence of the strong selective pressure exerted by ROS, even for those organisms that are typically found in microaerophilic or even anaerobic environments (40). Expression of these and related defensive enzymes is typically regulated by redox-sensing transcription factors. The two best characterized peroxide-sensors in the bacteria are *Escherichia coli* OxyR and *Bacillus subtilis* PerR (6, 23). Orthologs of one (and rarely both) of these H_2O_2-sensors are found in most bacteria and mediate the adaptive response to peroxides in which exposure to low levels of stressor enables survival when cells are subsequently faced with much higher levels of oxidant.

The ability of cells to elaborate defensive enzymes to degrade H_2O_2 is of paramount importance. For example, a mutant *E. coli* strain lacking both catalases and peroxidases (a hydroperoxidase minus or Hpx$^-$ strain) is compromised in its ability to grow under aerobic conditions even in the absence of any exogenous challenge with H_2O_2 (83). Increasingly,

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however, it is appreciated that the adaptive responses to ROS are far more nuanced than simply inducing the synthesis of defensive enzymes. Here, we focus specifically on the many ways in which bacterial cells, upon sensing H_2O_2, modify metal homeostasis to minimize the damage inflicted upon the cell.

Managing the Uneasy Liaison Between H_2O_2 and Metal Ions

Although H_2O_2 is quite chemically stable in simple solutions, it reacts rapidly with ferrous iron to generate highly reactive hydroxyl radical (*OH), hydroxide anion ($^-$OH), and oxidized ferric iron (Fenton reaction). Hydroxyl radical reacts at diffusion-limited rates with many biomolecules, including DNA. Cell death due to H_2O_2 is primarily due to DNA damage mediated by hydroxyl radicals, likely generated by reaction with ferrous iron loosely associated with DNA (Fig. 1).

Most cells require iron as a cofactor for enzymes, including some of the very enzymes needed to help protect against ROS (*e.g.*, heme-containing catalases and Fe-containing SOD) (40). Therefore, cells normally maintain a pool of free iron, presumably chelated by small molecules and protein chaperones, that serves as a reservoir for incorporation into newly synthesized metalloproteins. These cells face the paradox of needing to actively import an essential metal ion that can also, in the presence of ROS, catalyze destructive reactions. Moreover, ROS can further increase free iron levels by damaging metalloenzymes, including both mononuclear iron centers and Fe_4S_4 clusters (39). Reaction of H_2O_2 with iron centers can lead to protein damage *via* metal-catalyzed oxidation (MCO), whereas oxidation of Fe_4S_4 clusters can lead to disassembly

and loss of iron atom(s) from the cluster (42). For select groups of bacteria, including some lactobacilli and *Borellia burgdorferii*, this problem is avoided altogether by dispensing with a metabolic requirement for iron (3, 75).

In addition to reduced metal centers, H_2O_2 can also react with the thiolate anion including the active site cysteines of some cysteine-dependent enzymes and regulators. The rate constants for oxidation of thiolates by H_2O_2 vary widely; many proteins are relatively resistant to oxidation, whereas some, such as *E. coli* OxyR and thiol-dependent peroxidases, have evolved highly reactive thiol groups (25). These proteins can function as thiol-dependent sensors (the cysteine-disulfide switch of OxyR) or as peroxidases that help detoxify H_2O_2.

Each of *E. coli* OxyR and *B. subtilis* PerR regulates adaptive responses to H_2O_2, yet they ultimately rely on distinct sensing mechanisms. OxyR lacks any bound metal centers and instead senses peroxides *via* thiol oxidation (51). As a result, the OxyR regulon is induced by either H_2O_2 or by chemicals that oxidize and thereby deplete cellular thiol reductants (disulfide stress). In contrast, PerR senses both H_2O_2 and cellular metal status. PerR represses transcription when associated with either Mn(II) or Fe(II), but only the Fe(II)-containing form of the repressor can sense H_2O_2 (33, 59). PerR bound with Fe(II) is inactivated by H_2O_2-dependent MCO (53). This realization provided one of the earliest hints that peroxide stress responses were intimately linked to metal ion homeostasis (17).

Work in numerous systems, several of which are reviewed here, has now established that modulation of cellular metal ion content and distribution is an important and widespread component of bacterial adaptive responses to H_2O_2. While the details differ between species, a number of general themes have emerged. Characterization of the OxyR and PerR
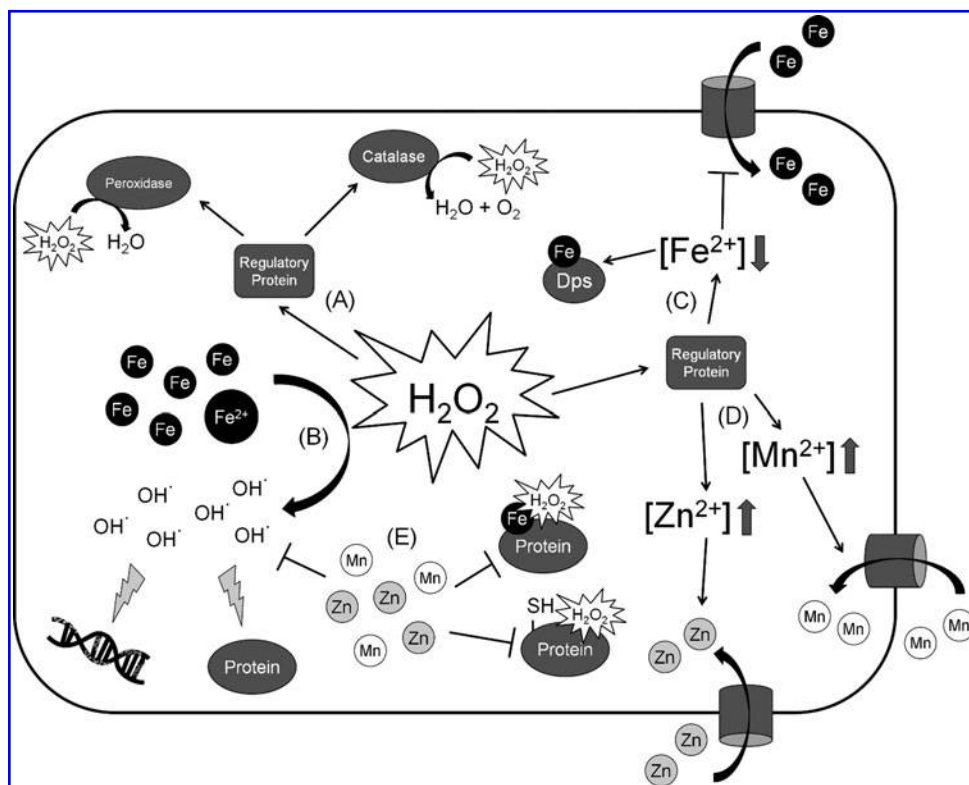

FIG. 1. General mechanisms for protection against oxidative stress. (A) To detoxify hydrogen peroxide (H_2O_2), many bacteria upregulate the expression of peroxidases and catalases. (B) H_2O_2 reacts rapidly with ferrous iron, generating hydroxyl radical, hydroxide anion, and oxidized ferric iron (Fenton reaction). The hydroxyl radical can then subsequently damage DNA and oxidize proteins. To protect against these toxic effects, bacteria may (C) decrease intracellular iron levels by decreasing Fe import and sequestering free iron through the upregulation of Dps. (D) Bacteria may also increase intracellular levels of Mn(II) and/or Zn(II) through increased import. (E) Mn(II) and Zn(II) can competitively inhibit the damaging reactions catalyzed by Fe(II) and H_2O_2.

regulons in a wide range of organisms suggests that peroxide stress elicits significant changes in cytosolic metal composition with the ultimate goal of reducing the levels of free, reactive ferrous iron and increasing the concentration of nonreactive divalent metals that can, by competitive binding, help prevent the adventitious association of Fe(II) with sensitive targets (Fig. 1). Iron homeostasis is modified by one or more of the following pathways: (i) repression of iron uptake (96), (ii) sequestration of cytosolic iron in ferritin and miniferritin (Dps family) proteins (2), and (iii) incorporation of iron back into newly synthesized or damaged Fe_4S_4 cluster enzymes (102). In parallel, metals such as Mn(II) and Zn(II) that can competitively inhibit the damaging reactions otherwise catalyzed by iron can be actively imported by the expression of peroxide-inducible, metal uptake systems (Fig. 1).

E. coli and the OxyR Regulon

E. coli OxyR is the prototype for a widely distributed family of redox-sensors (104). OxyR (and its orthologs) controls the adaptive response to H_2O_2 in many Gram-negative (and some Gram-positive) bacteria and is characteristic of the gamma and beta proteobacteria (61). OxyR is a tetrameric, DNA-binding transcriptional activator of the LysR family and binds upstream of the target genes within its regulon. Upon activation by H_2O_2, an intramolecular disulfide forms within each OxyR protomer, and the resulting conformational change enables productive interactions with RNA polymerase, and thereby leads to activation of the OxyR regulon (Fig. 2). In *E. coli* strains containing a full complement of peroxide-degrading enzymes (catalases and the alkyl hydroperoxide reductase AhpCF), activation of the OxyR regulon is commonly achieved by addition of millimolar levels of H_2O_2 although $5\,\mu M$ H_2O_2 will suffice (8). Additionally, studies with strains devoid of catalases/peroxidases (Hpx$^-$) indicate that OxyR induction commences once intracellular levels of H_2O_2 rise to $>100\,nM$ (83). Consistent with this, *in vitro* ~ 50–$200\,nM$ H_2O_2 oxidizes 50% of the OxyR protein (8). Indeed, OxyR is one of the most sensitive peroxide sensors known that can be attributed to its highly reactive active-site thiolate (87).

The OxyR regulon has been extensively documented and includes the major vegetative catalase, AhpCF, Dps, MntH, ferric uptake regulator (Fur), and the OxyS regulatory sRNA. When initially described in 1985, OxyR and its regulon was thought to be largely unrelated to metal homeostasis. The first hint that OxyR might affect iron homeostasis emerged with the realization that Dps, a known OxyR target gene (1), is related in both sequence (73) and structure to ferritin iron storage proteins (30). Although *E. coli* Dps can also bind and coat DNA, recent evidence suggests that it is the iron sequestration activity that is most important for protecting cells against peroxide-mediated damage (72). The induction of one or more iron sequestration proteins is one of the most widely distributed adaptations in response to peroxide stress, although the relevant regulatory pathways are varied.

In addition to iron sequestration, peroxide stress also increases the expression of the *E. coli* Fur protein (103). Fur is the master regulator of iron homeostasis and binds reversibly to Fe(II) to repress the expression of iron uptake functions (54). Fur also acts indirectly, *via* the RyhB sRNA (58) or by antagonizing H-NS mediated repression (63), to positively regulate the expression of iron-containing enzymes and storage functions. The ability of Fur to repress iron uptake when cytosolic levels of iron are elevated is of course adaptive and helps protect against H_2O_2-catalyzed Fenton chemistry. A mutant strain lacking Fur has elevated intracellular levels of "free" (chelatable) Fe(II) [*e.g.*, an increase from ~ 10 to $\sim 70\,\mu M$ in (47)] and a corresponding increase in H_2O_2 sensitivity. This

FIG. 2. The OxyR and ferric uptake regulator (Fur) regulons in *Escherichia coli*. **(A)** Upon exposure to H_2O_2, an intramolecular disulfide bond forms within each OxyR protomer, resulting in the activation of OxyR. Oxidized OxyR activates the sequestration of Fe (Dps), the detoxification of H_2O_2 (AhpC, KatG), and the import of Mn(II) (MntH). **(B)** OxyR also activates the transcription of the OxyS sRNA, resulting in the reduction of carbon utilization pathways and thus reducing endogenous H_2O_2 production. In addition, OxyR upregulates the synthesis of Fur. In Fe-rich conditions, **(C)** Fur represses additional Fe uptake

and **(D)** indirectly activates some iron-containing proteins (including Fe-superoxide dismutase [SOD] and bacterioferritin [Bfr]) *via* the RyhB sRNA or, in the case of the FtnA iron storage protein, by reversing H-NS-mediated silencing (63). In these conditions, the Isc Fe-S cluster biogenesis machinery is upregulated. Fur:Fe is sensitive to H_2O_2 and can be inactivated upon exposure. **(E)** However, in Fe-limited and/or oxidative stress conditions, transcription of the second Fe-S cluster assembly machinery, Suf, is favored.
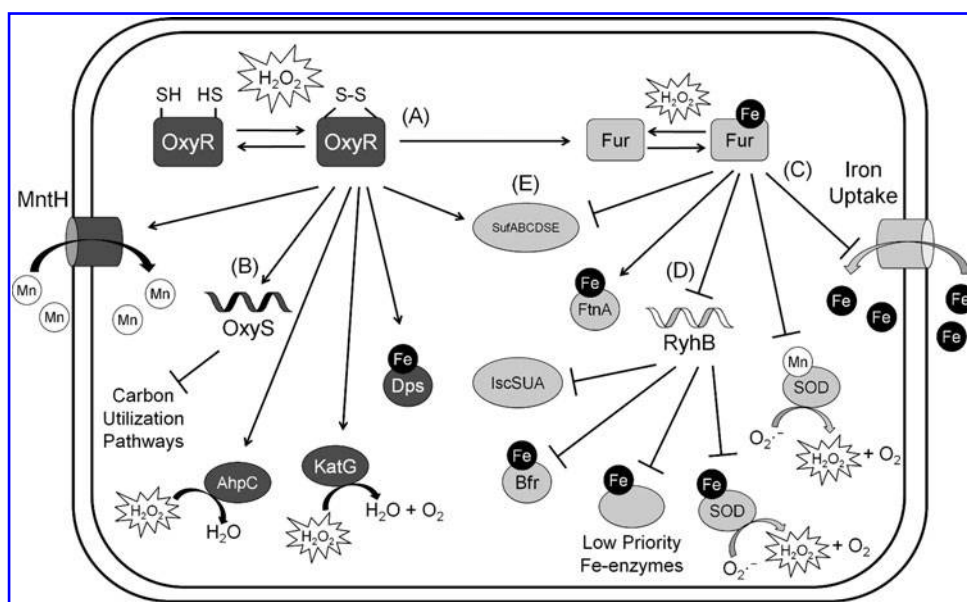
H_2O_2 sensitivity is largely iron dependent and can be reversed by a cell-permeable iron chelator. It has also been noted that H_2O_2 may inactivate *E. coli* Fur, possibly by oxidation of the bound Fe(II) and/or by MCO of the protein (96). This may be mechanistically similar to the pathway of MCO of *B. subtilis* PerR (53). In contrast, *B. subtilis* Fur is relatively insensitive to MCO (53, 54). In light of the studies cited above, however, the inactivation of *E. coli* Fur by MCO is unlikely to be adaptive and, in fact, may even sensitize cells to peroxides.

The physiological relevance of Fur induction by OxyR has several possible components. Fur itself is quite abundant for a regulatory protein with ∼5000 molecules per cell. This doubles under oxidative stress to ∼10,000 molecules per cell (103). Presumably, this serves to increase repression of the Fur regulon when sufficient iron is available. Alternatively, Fur may sequester iron by direct binding. Finally, induction of Fur may also serve to replace Fur proteins damaged by MCO. Support for this latter model has been provided by analysis of Fur function in strains lacking Hpx$^-$ (96). In this background, the Fur regulon is constitutively expressed during aerobic growth in minimal medium, presumably due to MCO of Fur by endogenously produced H_2O_2, which accumulates to 0.5–1.0 μM under these conditions. In rich medium, there is sufficient iron available that Fur repression can be restored, but only if OxyR can upregulate Fur synthesis (96).

In addition to regulating iron sequestration (Dps) and synthesis of the Fur metalloregulator, H_2O_2 also regulates the metallation of Fe-S cluster containing proteins. *E. coli* contains two parallel systems, encoding by the *isc* and *suf* operons, for the assembly and insertion of Fe-S clusters into proteins. In general, the Isc system serves a housekeeping role and is feedback regulated by an Fe-S containing repressor, IscR. IscR is a transcription factor encoded as part of the *iscRSUA* operon where IscS, IscU, and IscA are involved directly in the biogenesis and insertion of Fe-S clusters. When the activity of the Fe-S cluster machinery is sufficient, IscR contains an Fe_2S_2 cluster and represses the *isc* promoter (82).

Under conditions of iron limitation (67) or H_2O_2 stress (43), the Suf system can functionally substitute for the Isc system. The Isc system in disabled by low levels of H_2O_2, apparently due to oxidative inactivation of nascent Fe-S clusters (43). Under these conditions, activation of the *sufABCDSE* operon by OxyR is required for the efficient synthesis of Fe-S containing enzymes (43). Regulation of the *suf* operon by Fur, and activation by apo-IscR, contributes to the ability of this system to substitute for the Isc when iron is limited (55, 64, 67).

The RyhB sRNA also contributes to the regulation of Fe-S cluster assembly. In Fe-limited conditions, the Fur-regulated sRNA RyhB triggers the processing of the *iscRSUA* mRNA and subsequent degradation of the downstream portion of the mRNA (*iscSUA*). The result is that, in response to Fe-limitation, the Isc machinery is downregulated, but the IscR protein, required for the full activation of the *suf* operon, is still produced (21).

In addition to Fe(II), OxyR also has a significant impact on Mn(II) homeostasis (Fig. 2). OxyR activates the expression of *mntH* encoding a proton-dependent Mn(II) import channel (46). The resulting increase in intracellular Mn(II) is clearly adaptive since a strain defective in this Mn(II) uptake pathway has an increased sensitivity to H_2O_2. At least two mechanisms for this effect can be entertained. First, Mn(II) may function by virtue of its chemical activity since Mn(II),

particularly when complexed with bicarbonate, has been shown to have catalase-like activity and Mn(II) has been postulated to act as a quencher of damaging free radicals (57). Second, Mn(II) may act *via* chemical competition and simply displace Fe(II) bound to sites (*e.g.*, DNA and Fe-metalloproteins) where, if present, reaction with peroxides would lead to damaging MCO reactions. To distinguish between these models, Anjem *et al.* measured the rates for Mn(II)-dependent detoxification and concluded that, even with generous assumptions, the chemical activity of Mn(II) would pale besides the cell's enzymatic activity and would therefore be unlikely to contribute to adaptation (4). Conversely, they show that increased levels of intracellular Mn(II) decrease the chemical oxidation of many different cytosolic proteins and enzymes. These results provide strong evidence that Mn(II) plays a role as an antioxidant largely *via* chemical competition rather than direct chemical activity (Fig. 1). In *E. coli*, Mn(II) import is conditionally evoked in response to peroxide stress (4), whereas in other bacteria relatively high ambient Mn(II) levels are maintained even in the absence of overt stress (18, 65, 71).

B. subtilis and the PerR Regulon

The adaptive response to H_2O_2 and metal ion homeostasis are also intricately linked in *B. subtilis* (Fig. 3). Indeed, the discovery of PerR, and the molecular genetic analysis of peroxide adaptation in this system evolved from studies designed to monitor changes in gene expression in response to metal deprivation (16). In these early studies, gene fusions were sought that were derepressed under conditions of iron starvation using the chelator EDDHA. Characterization of the resulting metal-regulated genes led to the identification of *mrgA*, encoding a Dps family protein. Like Dps, MrgA is a bacterial mini-ferritin that can both sequester iron and bind nonspecifically to DNA (15). Characterization of *mrgA* led to the realization that this gene was regulated both by metal ions (induced by metal starvation) and by H_2O_2. Moreover, both types of regulation required the same *cis*-acting sequence subsequently shown to be the binding site of the PerR repressor (and therefore designated as a Per box) (17). PerR, a Fur family member, is a metal-dependent DNA-binding protein (11, 33, 53). These same studies also led to the identification of two other paralogs: Fur (the *bona fide* iron uptake regulator) and Zur (a zinc uptake regulator) (54).

B. subtilis PerR is the prototype for a widely distributed family of H_2O_2-sensing metalloproteins. PerR homologs are found in many Gram-positive bacteria, particularly within the Firmicutes, but are also found in *Campylobacter* spp. (95) and the cyanobacteria (61). PerR is a dimeric, DNA-binding protein containing, within each monomer, a tightly held structural Zn(II) (52) and a loosely associated regulatory metal ion (53). Under conditions of metal deficiency, such as growth in minimal medium with very low levels of iron and manganese, PerR is inactive as a repressor (this inactive metalloprotein is designated PerR:Zn). In most rich medium conditions, PerR binds Fe(II) as corepressor (PerR:Zn,Fe) and this form of the protein represses the PerR regulon. H_2O_2 leads to MCO of the protein in which the bound Fe(II) reduces H_2O_2 to generate a hydroxyl radical (Fenton reaction), which, in turn, modifies either one of two specific histidines coordinated to the iron atom (53). The resulting inactivated protein, containing a single 2-oxo-histidine residue, dissociates from DNA leading

FIG. 3. The PerR, Fur, and MntR regulons in *Bacillus subtilis*. **(A)** H_2O_2 oxidizes PerR:Fe, resulting in the derepression of peroxide detoxifying enzymes (KatA and AhpCF), an Fe-sequestration protein (MrgA), heme biosynthesis enzymes, and Zn(II) uptake. Although PerR:Mn can also repress these same genes, this form of PerR is relatively insensitive to oxidation by peroxide. Therefore, in low Fe but high Mn conditions, the PerR regulon is not derepressed upon exposure to peroxides. **(B)** Only PerR:Mn, not PerR:Fe, appears to be responsible for the repression of Fur. **(C)** In Fe-limited conditions, iron uptake is derepressed by Fur. **(D)** Production of low priority Fe-enzymes is decreased by the FsrA sRNA when Fe is limiting. **(E)** Independent of Fur and PerR activity, MntR represses Mn(II) uptake when Mn(II) is sufficient.
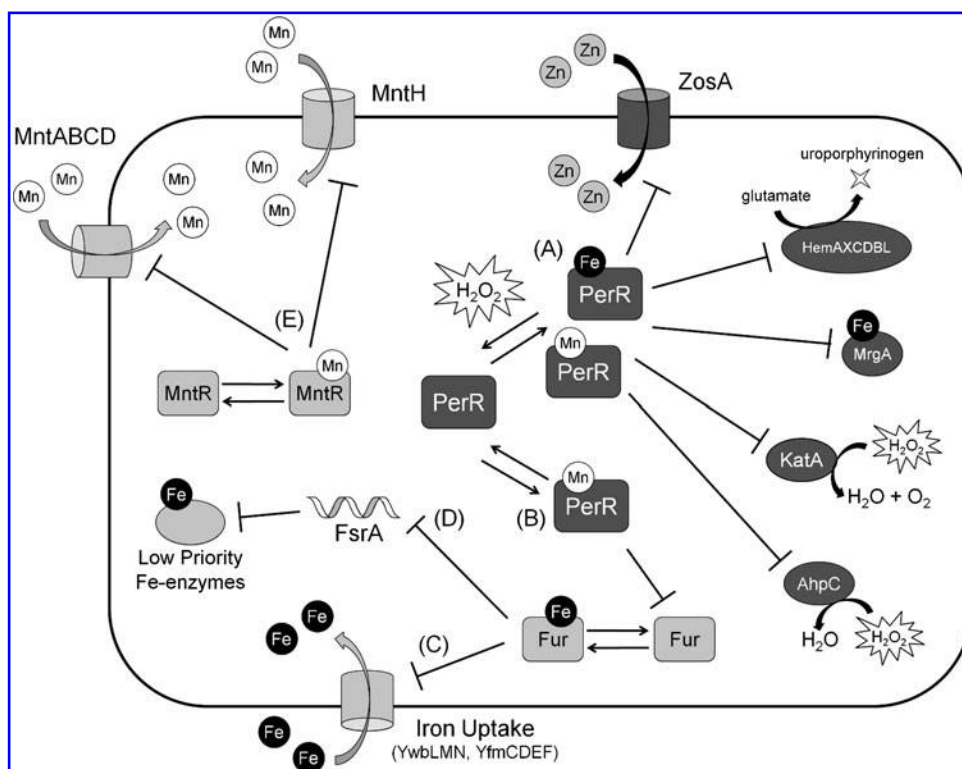
to derepression of the regulon (53, 90). This modification has been observed, and the regiospecificity rationalized, by analysis of the structure of PerR in both its oxidized and reduced states by X-ray crystallography (23).

PerR can also repress gene expression when associated with other regulatory metal ions: a phenomenon studied in most detail for Mn(II) (16). The PerR:Zn,Mn form of the repressor, however, is refractory to H_2O_2 and, under these conditions, the PerR regulon is largely repressed and noninducible (26). Thus, PerR can be thought of as a signal integrator that simultaneously monitors both H_2O_2 levels and the ratio of cytosolic Mn(II) to Fe(II). Under conditions of relatively high Mn(II), and low Fe(II), many cytosolic proteins and enzymes may be relatively protected against protein-damaging MCO reactions, as noted above for *E. coli*, and thus induction of the PerR regulon may not be necessary. Indeed, genetic studies suggest that full induction of the PerR regulon is deleterious to the cell; a *perR* null mutant strain grows very slowly and has defects that can be, at least partially, explained by aberrant regulation of iron homeostasis (our unpublished results).

B. subtilis contains both PerR and Fur proteins ($\sim 30\%$ identical), but the two differ dramatically in their sensing properties. PerR exists largely in the PerR:Zn,Fe form in cells grown in Fe-sufficient medium, but in medium limited for iron and with sufficient Mn(II) PerR exists instead in the PerR:Zn,Mn form. Fur binds Fe(II) with somewhat higher affinity than PerR and is poised to sense severe drops in cytosolic iron levels and to respond by derepression of iron uptake pathways (and translational repression of many iron-utilizing enzymes through the FsrA sRNA) (27, 66). PerR:Zn,Fe reacts rapidly with H_2O_2 by MCO and is thus poised to sense submicromolar levels of H_2O_2 in the cell. In contrast, Fur:Zn,Fe is comparatively H_2O_2 resistant and does not readily undergo MCO (53). This contrasts with the situation in *E. coli* that has a single protein (Fur) that senses Fe(II) but can also be inactivated by H_2O_2, in an apparently maladaptive process (96). Defining how Fur family proteins can tune their selectivity for metal ions, and their reactivity with peroxides when bound with Fe(II) remains a challenging problem for further study (54).

Derepression of the PerR regulon leads to a large increase in peroxide resistance (11). This can be attributed, in part, to derepression of enzymatic detoxification systems, including the major vegetative catalase (KatA) and alkylhydroperoxide reductase. Coincident with derepression of KatA, which becomes one of the most abundant cellular proteins in a *perR* null mutant strain, there is derepression of heme biosynthesis genes, presumably to support the high level of catalase activity. Derepression of MrgA is hypothesized to decrease the levels of free Fe(II) within the cell by sequestration within the dodecameric (mini-ferritin) protein shell. As a class, Dps family proteins often help protect cells against ROS and increase survival in stationary phase (2). Stationary phase is not always well defined, but anytime cells must maintain genome integrity over extended periods of time, often under conditions of energy depletion, it is especially important to minimize DNA damage. Dps family proteins appear to protect DNA primarily by their ability to sequester Fe(II), but the ability of many family members to also bind directly to DNA (to form intracellular nanocrystals) (30) may also play a role.

Many bacteria contain multiple Dps orthologs, although the reason is not always clear. In *B. subtilis*, one Dps ortholog (encoded by *dps*) is regulated by the σ^B general stress response

σ factor, induced upon entry into stationary phase, and plays a major role in the high intrinsic resistance of stationary phase cells to H_2O_2 (5). The second, MrgA, is induced as part of the PerR-regulated peroxide-stress response and also serves to protect cells against ROS-mediated damage (15). The biochemical and functional differences between these two paralogs are not yet well defined. *B. anthracis* also encodes two paralogs that differ in their preferred oxidant for iron mineralization. Both Dps1 and Dps2 mineralize Fe with molecular oxygen as the oxidant, whereas only Dps2 functions efficiently with H_2O_2. Dps2, considered the MrgA ortholog, may therefore function primarily to reduce cytosolic iron levels in response to H_2O_2 stress, whereas Dps1 may sequester iron when in excess. Indeed, it has been suggested that, as a class, Dps proteins may serve primarily in preventing iron-mediated Fenton chemistry; it is not clear if the mineralized iron core can be mobilized when iron is needed. In contrast, ferritins and bacterioferritins are thought to play a dominant role for iron storage (2).

In addition to its regulation of *mrgA*, PerR may affect metal ion homeostasis in other ways. The *fur* gene itself is a member of the PerR regulon although, intriguingly, the repression of Fur by PerR is mediated only by the PerR:Zn,Mn form of the repressor. As a result, and in contrast to *E. coli*, transcription of *fur* seems not to be inducible by H_2O_2 (26). Instead, PerR may regulate Fur expression in response to the relative levels of Fe(II) and Mn(II) within the cell (26).

PerR also represses a metal-transporting P-type ATPase known as ZosA (zinc uptake under oxidative stress) (28). Physiological studies have led to a model in which ZosA imports Zn(II) in response to oxidative stress (Fig. 3), although the actual specificity of this transporter has not yet been rigorously investigated. The proposed function of imported Zn(II) is to displace Fe(II) from sensitive sites in proteins or on the surface of DNA and thereby to function as an antioxidant *via* a chemical competition mechanism analogous to that discussed above for Mn(II) (28). Interestingly, and unlike the situation in *E. coli*, there is no evidence for derepression of *mntH* by ROS in *B. subtilis*. It is unclear why some bacteria import Mn(II) and others choose Zn(II) for the purposes of protecting against the deleterious actions of cytosolic Fe(II).

The regulatory protein responsible for maintaining Mn homeostasis in *B. subtilis* is MntR, which belongs to the diphtheria toxin repressor (DtxR) family of metalloproteins (60). DtxR was originally discovered as an iron-dependent repressor of toxin production. However, it now appears that DtxR homologs have a broader role and may regulate iron and/or manganese homeostasis. An *mntR* mutant strain is more sensitive to Mn(II) relative to wildtype, suggesting a role for MntR in controlling Mn transport. Consistent with this, in the presence of Mn(II), MntR represses expression of MntH and an Mn(II) ABC transporter encoded by *mntABCD*. Mutation of *mntH* in a *mntR* mutant strain restores partial resistance to Mn(II), suggesting that MntH is the major pathway of Mn(II) uptake when Mn(II) levels are high (77). An *mntR* mutant strain has an increased sensitivity to H_2O_2. This sensitivity is not due to the derepression of *mntH* since an *mntR mntH* double mutant is also H_2O_2 sensitive. One possibility is that derepression of the *mntABCD* metal uptake system leads to an increase in iron uptake. Indeed, orthologous ABC transporters import both Mn(II) and Fe(II) (20, 49, 79, 80).

The PerR-regulated response to oxidative stress described for *B. subtilis* and its connection to metal ion homeostasis provide a model for understanding the complex responses employed by several other bacteria. One such example is found in *Staphylococcus aureus*. The PerR-regulated response for *S. aureus* resembles closely that of *B. subtilis* except in the interplay between PerR activity and iron levels (36). As in *B. subtilis*, PerR functions as a repressor for genes of the peroxide stress response and this activity appears to require a bound metal cofactor. In both systems, Mn(II) is an efficient corepressor, but, unlike the situation in *B. subtilis*, high iron levels lead to derepression of the PerR regulon in *S. aureus*. Since the PerR regulon genes are inducible by peroxide, it seems most parsimonious to suggest that high iron leads to ROS production and inactivation of the PerR:Zn,Fe form of the repressor, thereby leading to apparent induction by iron. Alternatively, PerR in this system may function as an Mn(II)-specific repressor (analogous or equivalent to Mur) (54) and iron may function as an antagonist of its DNA-binding activity. In this scenario, peroxide stress would be sensed indirectly as an increase in free iron levels. To complicate matters further, there is an incompletely understood interplay between the PerR and Fur regulons in *S. aureus* (37). For example, catalase in this system is repressed by PerR in response to Mn(II) and activated by Fe(II), directly or indirectly, by Fur. These results are generally consistent with the theme that high Mn(II) levels are protective against ROS and that induction of catalase and other defensive enzymes may therefore be less important under these conditions.

Although best characterized in members of the low GC Gram-positive bacteria (*Firmicutes*), PerR has also been described in more distantly related bacteria. One notable example is the Gram-negative, foodborne pathogen *Campylobacter jejuni* (95). This organism contains both Fur and PerR orthologs that together coordinate the regulation of iron homeostasis functions and peroxide stress responsive genes. As in *B. subtilis*, PerR mediates an iron-dependent repression of catalase and AhpCF in this organism. Recent transcriptome analyses have investigated these responses in some detail (68), but some of the findings seem to contradict prior work, suggesting that additional studies will be required to better understand the complexities of regulation in this system.

The *E. coli* OxyR (Fig. 2) and *B. subtilis* PerR regulons (Fig. 3) provide important touchstones for thinking about the complex relationships between peroxide stress responses and metal ion homeostasis. The general themes developed from analysis of these systems inform ongoing studies of stress responses in numerous other systems. Here, we focus specifically on four diverse examples: *Deinococcus radiodurans*, *Neisseria gonorrhoeae*, *Streptococcus pyogenes*, and *Bradyrhizobium japonicum.*

Deinococcus radiodurans

D. radiodurans is known for its extreme resistance to ionizing and ultraviolet radiation. The resistance of *D. radiodurans* to radiation correlates with the ability of this organism to combat oxidative stress, since the lethal effects of radiation can be largely attributed to radiolysis of water leading to hydroxyl radical and H_2O_2, which, in turn, drives MCO of proteins and nucleic acids (18). Initially, it was suspected that *D. radiodurans* might possess unique DNA repair mechanisms

or pathways that could account for its remarkable ability to re-assemble its chromosome and thereby recover from conditions leading to multiple double-stranded breaks. More recent results have highlighted instead a key role for metal ion homeostasis, and in particular, a high Mn:Fe ratio in protecting proteins (including the DNA repair machinery) against ROS-mediated inactivation (18).

Multiple factors regulate the expression of the genes integral to the *D. radiodurans* oxidative stress response (Fig. 4), including OxyR, a PerR/Fur homolog, and DtxR. OxyR is unique in that it has only one cysteine residue (C210) in contrast to the two redox-active cysteine residues present in the prototypical *E. coli* OxyR (14). Upon exposure to peroxides, C210 becomes oxidized to a sulfenic acid. This cysteine residue does not appear to form an intermolecular disulfide bond with another OxyR subunit, and it is not known if it becomes further oxidized. In addition to OxyR, *D. radiodurans* has a homolog belonging to the PerR/Fur family of proteins; however, little is currently known on its sensing mechanism and regulon. A third regulator is DtxR, which is important for the regulation of both iron and manganese homeostasis (13). Collectively, these three proteins coregulate oxidative stress responses and metal homeostasis in *D. radiodurans*. The regulation of catalase (*katE*) highlights the interplay between the regulation by these three proteins. The expression of *katE* is activated by oxidized OxyR (14). Additionally, its expression may be repressed by both DtxR and the PerR/Fur homolog; *katE* is derepressed in both a *dtxR* mutant strain and a *perR/fur* mutant strain (13, 14). Interestingly, *D. radiodurans* has one of the highest catalase activities reported for any bacterium (97), which may account for the complex regulation of the *katE* gene.

One of the most striking features of *D. radiodurans* is its very high intracellular manganese to iron ratio (0.24 for *D. radiodurans* compared to 0.0072 for *E. coli*). A high Mn:Fe ratio correlates with a high resistance to ionizing radiation-induced protein oxidation in bacteria. An ionizing radiation dose of 12,000 gray (absorbed radiation dose, Gy) decreases the number of viable *D. radiodurans* cells by 90% (D_{10}). In comparison, the D_{10} for *E. coli* is 700 Gy (18), indicative of a strong correlation between intracellular Mn:Fe ratio and resistance to radiation and ROS. For example, *Enterococcus faecium* has a high intracellular Mn:Fe ratio (0.17) and, accordingly, has a high resistance to ionizing radiation ($D_{10} = 2000$ Gy), whereas *Shewanella oneidensis* and *N. gonorrhoeae* have low Mn:Fe ratios (0.0005 and 0.0004, respectively) and are not very resistant to ionizing radiation ($D_{10} = 70$ and 125 Gy) (19).

Consistent with the proposed importance of the Mn:Fe ratio, import of Mn appears to be inversely correlated with the import of Fe and both are under complex regulation. Expression of the *D. radiodurans* Mn and Fe transport proteins are coregulated by OxyR and DtxR (13, 14). *D. radiodurans* has two Mn(II) transporters (MntH and MntABCD) and at least two Fe transporters: an Fe(II) transport protein B (*feoB*) and an Fe(III) dicitrate-binding protein (drB1025). DtxR is proposed to activate transcription of the two iron transport genes while repressing the expression of the MntABCD Mn(II) transporter (13). In addition, OxyR may regulate the expression of the Fe transport proteins (14) and also activate expression of MntH, as observed for *E. coli*. A strain mutated in either *oxyR* or *dtxR* has an increased Mn:Fe ratio (0.321 for wildtype, 0.498 for the *oxyR* mutant, and 0.445 for the *dtxR* mutant) (13, 14). In addition to the iron transport proteins, expression of one of two

FIG. 4. The OxyR and DtxR regulons in *Deinococcus radiodurans*. **(A)** *D. radiodurans* OxyR has only one redox active cysteine residue. This cysteine residue is oxidized upon exposure to H_2O_2. OxyR may regulate Mn and Fe homeostasis; however, the details of this regulation are not yet clearly defined. **(B)** Catalase activity is regulated by three regulatory proteins: OxyR, DtxR, and a PerR/Fur homolog. This tight regulation may relate to the high level of catalase activity present in this bacterium. **(C)** In addition to OxyR, DtxR also regulates Mn and Fe import. Mn transport is repressed under conditions where Fe transport is induced. Direct effects are shown as *solid lines* and potentially indirect effects are shown as *dotted lines*.
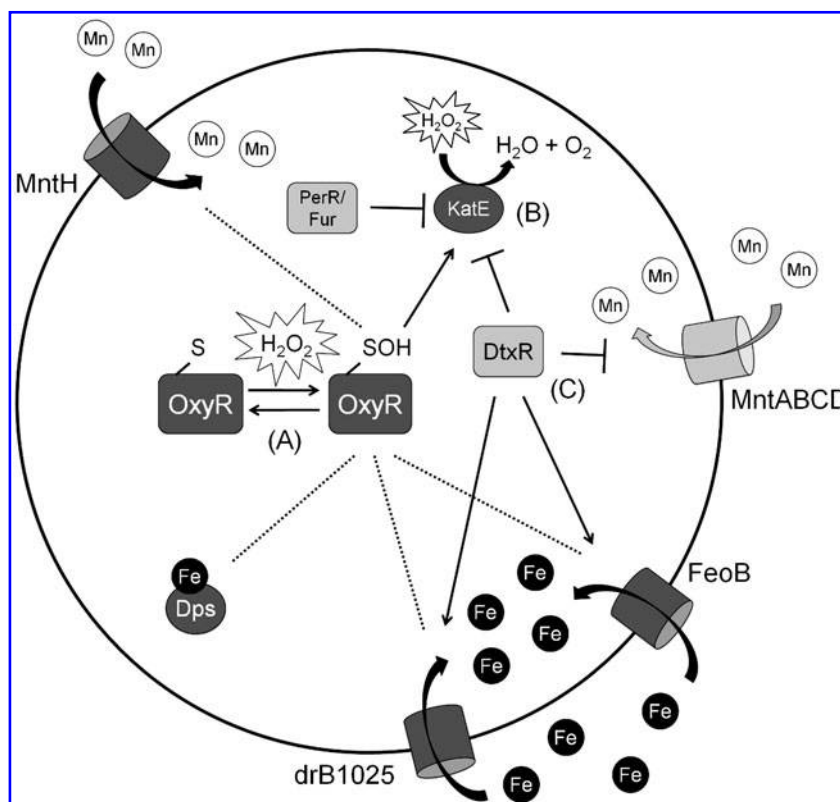
dps genes in *D. radiodurans* is in part regulated by OxyR. However, this does not appear to be the dominant form of regulation since exposure of an *oxyR* mutant strain to H_2O_2 results in a very large upregulation of *dps* (about 120 times that of wildtype without H_2O_2), indicating that additional factors, yet unknown, likely regulate expression of *dps* (14). The studies reported to date in *D. radiodurans* indicate that this organism relies heavily on a well-coordinated modulation of metal ion homeostasis, ultimately affecting the Mn:Fe ratio within the cell, as a primary mode of defense against conditions leading to reactive radicals (oxidative stress and ionizing radiation) (18). However, additional work is needed to decipher the precise manner in which these regulators (and those yet to be defined) coordinate metal ion homeostasis with oxidative stress responses.

Neisseria gonorrhoeae

Most bacteria have either OxyR or PerR to sense and respond to H_2O_2 (61). Thus, it was something of a surprise when *N. gonorrhoeae* was proposed to have both an OxyR and a PerR regulatory system (84, 98). PerR was originally defined in this system as an Mn(II)-sensing repressor and mutants lacking PerR were highly resistant to H_2O_2 (98). While these properties are reminiscent of *B. subtilis* PerR, the *N. gonorrhoeae* homolog differs in other ways. Specifically, it does not appear to sense peroxide nor does it regulate classic peroxide stress response genes such as catalase.

It seems likely that OxyR is the key regulator of the adaptive response to low level peroxide stress in *N. gonorrhoeae*. OxyR controls a small regulon of three genes encoding catalase (*katA*), peroxidase (*prx*), and a glutathione oxidoreductase (*gor*) (84). *N. gonorrhoeae* has ∼100-fold higher catalase activity than *E. coli* (7). An *oxyR* mutant strain of *N. gonorrhoeae* has elevated catalase activity and is more resistant than wildtype to H_2O_2 stress, which suggests that OxyR acts as a repressor of *katA* (91). More recent results indicate that OxyR likely functions as both a repressor (in the absence of stress) and peroxide-responsive activator of catalase (38). In addition, OxyR activates the expression of *prx*, encoding a peroxiredoxin, and a glutathione oxidoreductase (84).

The PerR regulon, as defined by transcriptomics, bears a remarkable similarity to the Zur regulon in other bacteria. Specifically, PerR represses an ABC transporter for metal ion import and two ribosomal protein paralogs (L31 and L36). Duplicated genes encoding ribosomal proteins are found in numerous bacteria, and, in all cases examined to date, one paralog is induced in response to zinc starvation (70). Induction serves, in most cases (*e.g.*, L31, L34, and L36), to mobilize zinc by displacement of the corresponding Zn-containing paralog from the surface of the ribosome. Alternatively, synthesis of a Zur-regulated, zinc-independent ribosomal protein (S14 paralog) enables continued ribosome synthesis in the absence of readily available zinc to metallate S14 (29, 62, 70). The ABC transporter regulated by PerR is annotated as MntABC and is involved in Mn(II) import, which plays an integral role in the oxidative stress resistance of *N. gonorrhoeae* (62). However, this transporter also mediates import of Zn(II), consistent with PerR functioning as a Zur ortholog (56). Further studies are needed to determine whether this PerR is formally analogous to Zur [a Zn(II)-sensing

repressor], Mur [an Mn(II)-sensing repressor], or perhaps both.

The role of metal ions, and in particular Mn(II), in the oxidative stress resistance of *N. gonorrhoeae* is well documented. When grown in medium supplemented with 100 μM Mn(II), cells are more resistant to oxidative stress than when grown in unsupplemented media. Addition of other cations, including Zn(II), had no protective effect (92). A strain mutant in *mntAB*, encoding the membrane-associated components of the ABC transporter, is severely compromised in its growth under nonstressed conditions (98). Addition of either Mn(II) or Zn(II) to the growth media restored good growth of an *mntC* mutant and MntC was found to bind both Mn(II) and Zn(II) with similar affinity (56). Collectively, these results suggest that the ability of *Neisseria* to maintain cytosolic pools of Zn(II) and/or Mn(II) is critical for resistance to ROS, although there is little evidence to date to suggest that uptake is induced by oxidative stress.

In addition to PerR, *N. gonorrhoeae* also contains a *bona fide* Fur that functions specifically in iron homeostasis (Fig. 5). Fur mediates the iron-dependent repression of iron acquisition genes, including transferrin-binding proteins (TbpAB), ferric enterobactin transport protein (FetA, formerly FrpB), and ferric binding protein system (FpbABC) (41). Additionally, *N. gonorrhoeae* activates a bacterioferritin (*bfrAB*) and an Fe-containing SOD (*sodB*) in Fe-rich conditions. A Fur-regulated small RNA named NrrF (Neisserial regulatory RNA responsive to iron) likely mediates the indirect activation of *bfrAB* and *sodB* by Fur (24, 41). Although the upregulation of Bfr in Fe-rich conditions suggests that it serves primarily as an Fe-storage protein, a *bfrB* mutant strain is more sensitive to both H_2O_2 and paraquat highlighting again the connection between iron homeostasis and oxidative stress resistance (12).

A complex picture of the adaptive response to H_2O_2 has emerged from a comparison of the peroxide stimulon (those genes induced by peroxide) and the regulons controlled by PerR, Fur, and OxyR (86). After 15 min of exposure to 5 mM H_2O_2, 75 genes were significantly upregulated and many of these were members of the Fur regulon (41). This suggests that, at least with these high levels of exogenous H_2O_2, the Fur repressor is inactivated. Unexpectedly, there appeared to be little induction of OxyR-regulated genes in this study. In contrast, exposure to 1 mM H_2O_2 did significantly upregulate the OxyR regulon (84). Moreover, H_2O_2-mediated induction of *N. meningitidis* catalase was found to be maximal with ∼135 μM H_2O_2, indicative of a sensitive and specific response as mediated by OxyR (38). These studies are consistent with the notion that OxyR serves as a first line of defense against low level H_2O_2 stress, whereas additional regulons are engaged at much higher levels of ROS.

Although the connections between the OxyR, PerR, and Fur regulons are poorly understood, there are some intriguing correlations. It has been noted, for example, that growth in iron-limiting conditions results in an increased expression of *oxyR* (41). OxyR regulates the expression of two peroxide detoxifying enzyme (KatA and Prx), only one of which uses iron. Whereas KatA uses a heme cofactor to reduce H_2O_2, Prx uses thiol-redox chemistry. One can speculate that increased expression of OxyR under iron-limiting conditions might serve to increase repression of the iron-requiring KatA while still allowing upregulation of Prx in the event of peroxide stress.

FIG. 5. The OxyR, PerR, and Fur regulons in *Neisseria gonorrhoeae*. (A) Oxidization of OxyR results in the upregulation of two H_2O_2 detoxifying enzymes, KatA and Prx. **(B)** Regulation of the oxidative stress response and Fe homeostasis are linked by the (potentially indirect) repression of OxyR by Fur:Fe. In addition, Fur may be inactivated by H_2O_2. In Fe-limited conditions, Fe uptake is derepressed and **(C)** Fe storage is repressed by the NrrF sRNA. NrrF is also responsible for the repression of an Fe-SOD. **(D)** In addition to OxyR, *N. gonorrhoeae* contains a PerR homolog that likely functions as a Zur (or possibly a Mur) regulatory protein. PerR is responsible for the repression of Mn and Zn uptake and a periplasmic peroxidase in Mn-replete (and possibly Zn-replete) conditions. Direct effects are shown as *solid lines* and potentially indirect effects are shown as *dotted lines*.
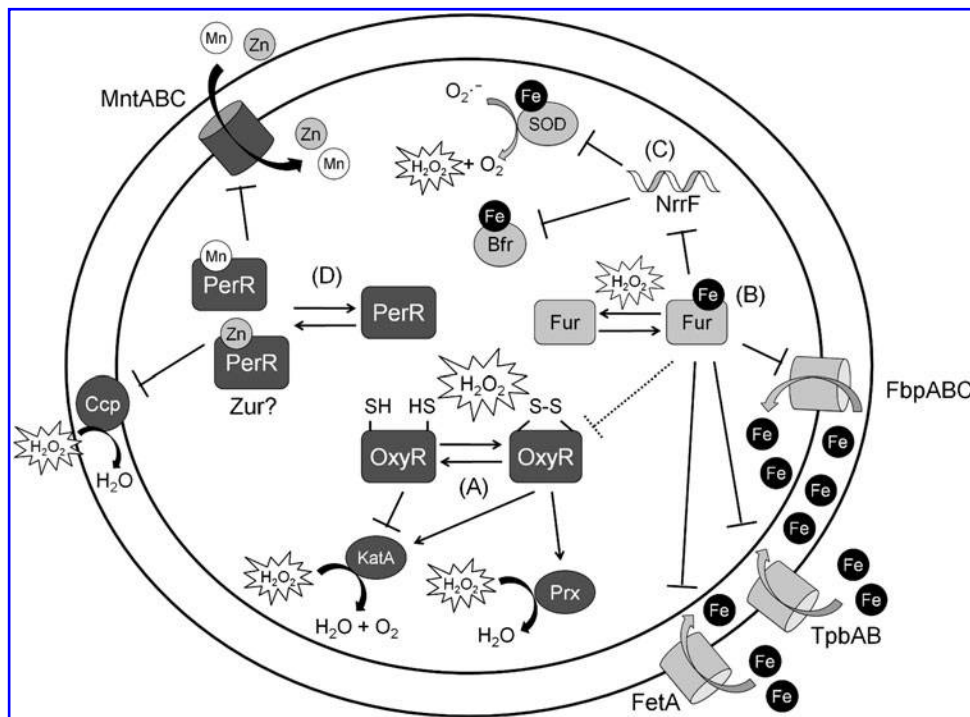
Streptococcus pyogenes

S. pyogenes, an important Gram-positive pathogen, lacks catalase and does not synthesize heme. In addition, the two known peroxidases in this organism, alkylhydroperoxide reductase (*ahpCF*) and glutathione peroxidase (*gpoA*), do not appear integral for protection against oxidative stress; a strain mutant in either *ahpC* or *gpoA* is not more sensitive to peroxide than wildtype nor is the expression of either *ahpC* or *gpoA* induced in conditions of oxidative stress (48). An *ahpC* mutant strain accumulates more endogenously produced peroxide than wildtype, suggesting a role for AhpC in the protection against low levels of H_2O_2, similar to that observed for *E. coli* (83). Although *S. pyogenes* expresses PerR, this regulatory protein does not control the expression of alkylhydroperoxide reductase or glutathione peroxidase (48). Due to this apparent lack of a strong direct defense against peroxides, it is of interest to explore the interplay between the oxidative stress response and metal homeostasis in *S. pyogenes* (Fig. 6).

As observed for *B. subtilis* PerR, PerR from *S. pyogenes* responds to H_2O_2 by the derepression of oxidative stress genes (94). Among the genes repressed by PerR, one encodes a ferritin-like protein belonging to the MrgA/Dps family and a second encodes PmtA, a CPx-type heavy metal transporter homologous to ZosA (10, 94). The MrgA ortholog in this genus was originally described in *Streptococcus mutans* and named Dpr (a Dps homolog) (100), although the *S. pyogenes* ortholog has been referred to as both MrgA (9) and Dpr (93, 94). Recall that both *mrgA* and *zosA* are repressed by *B. subtilis* PerR. Regulation by PerR has been studied in most detail for *dpr*, which is induced by elevated levels of H_2O_2 (0.5–1.0 m*M*), but constitutively expressed in a *perR* mutant (94). Expression

of *dpr* was also found to be elevated when cells were exposed to millimolar levels of metal ions, including Fe(II), Zn(II), and Ni(II). If PerR functions as described for *B. subtilis*, iron is the presumed PerR corepressor, which seems contrary to the observed induction by added iron. However, addition of high levels of Fe(II) to culture medium will likely lead to the formation of H_2O_2 *via* the reaction of Fe(II) with oxygen through Fenton chemistry, which may account for the observed derepression. The effects of Zn(II) and Ni(II) may reflect the ability of these metal ions to act as antagonists of metal-dependent DNA-binding by PerR.

Consistent with its regulation by PerR, Dpr likely confers protection against oxidative stress and this reflects its ability to chelate iron and thereby prevent Fenton chemistry (93). Indeed, addition of an iron chelator can rescue the peroxide sensitivity of a *dpr* mutant. The *dpr* mutant strain also does not grow as well in iron-limiting conditions and has a lower rate of survival in excess iron (30 m*M* ferrous sulfate) compared to wildtype. These results suggest that Dpr may also have physiologically significant roles in iron storage and sequestration (Fig. 6).

Although PmtA is homologous to ZosA from *B. subtilis*, a proposed Zn(II) uptake system (28), PmtA appears to be a metal efflux pump. A *perR* mutant strain, which has *pmtA* derepressed, has increased resistance to zinc toxicity (10). In contrast, a *perR* mutant strain of *B. subtilis* is more sensitive to zinc toxicity due to derepression of *zosA* (28). In support of the putative Zn(II) efflux activity of PmtA, derepression of PmtA is linked to the elevated expression of some genes regulated by AdcR, known to be induced by zinc starvation. AdcR also regulates the ABC transporter AdcABC implicated in the import of Zn(II), and possibly Mn(II), in *S. pneumonia* and *S. mutans* (22), but this operon was not induced in a *perR*
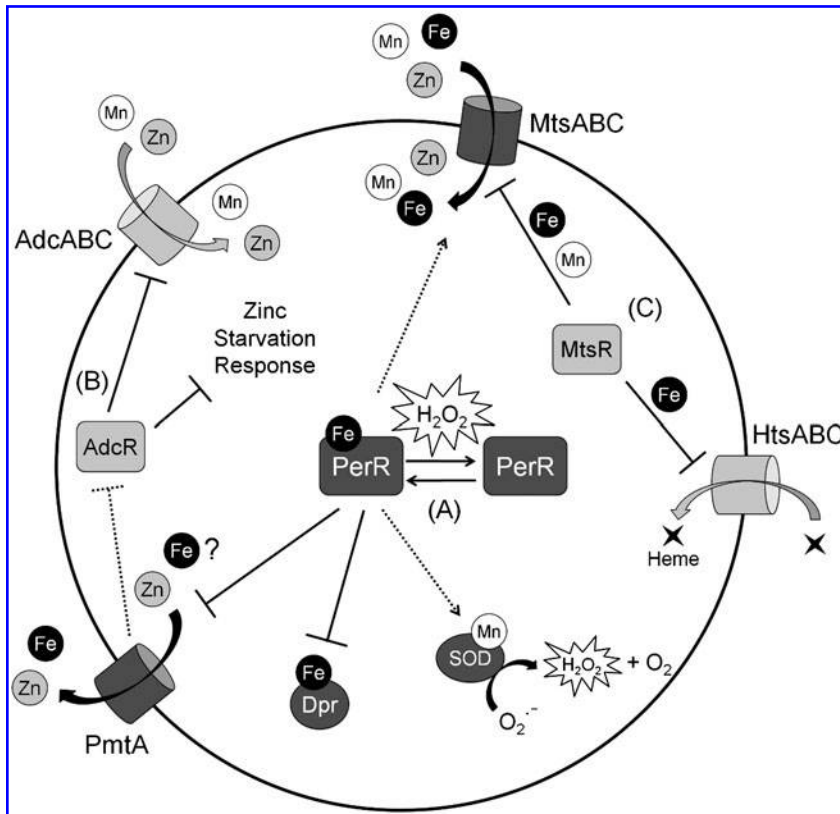
FIG. 6. The PerR regulon in *Streptococcus pyogenes*. (A) Upon exposure to peroxides, oxidized PerR derepresses iron sequestration (Dps) and cation export (Zn and possibly Fe) *via* PmtA. Import of Fe, Zn, and Mn may also be decreased upon exposure to H_2O_2 *via* the downregulation of MtsABC. **(B)** The combined effect of these alterations of cation import is the activation of the zinc starvation response through AdcR. **(C)** Mn, Fe, and heme homeostasis are additionally controlled by MtsR in a metal-specific manner; either Fe- or Mn-bound MtsR can repress MtsABC, but only MtsR:Fe can repress heme import. Direct effects are shown as *solid lines* and potentially indirect effects are shown as *dotted lines*.

mutant. Therefore, the link between derepression of *pmtA* and increased resistance to H_2O_2 remains unclear. Since other organisms increase the import of Mn(II) or Zn(II) in response to H_2O_2 stress, it seems counterintuitive that *S. pyogenes* would efflux Zn(II). Moreover, it is unclear how this might correlate with peroxide resistance. One possibility is that PmtA may also efflux Fe(II), and thereby reduce the incidence of Fenton chemistry within the cell. Indeed, accumulation of Fe, Mn, and Zn is decreased in the *perR* mutant strain of *S. pyogenes* (44, 78). The molecular basis for reduction in intracellular metal ion levels is presently unclear. In one study, decreased iron levels were correlated with reduced transcription of the ABC transporter MtsABC in the *perR* mutant (78). In addition to Fe(II), some studies suggest that MtsABC may import Mn(II) and/or Zn(II), but there are varying viewpoints on the physiological relevance of these observations (32, 44, 78, 89). Conversely, levels of *mtsA* were not reduced in the *perR* mutant in a second study (32). MtsR, an MntR ortholog, also regulates the expression of *mtsABC* together with the HtsABC heme importer (32).

PerR may also regulate, potentially indirectly, the expression of the single SOD (which is Mn-dependent) in *S. pyogenes*; a *perR* mutant has reduced transcription of *sodA* and is more sensitive to superoxide ($O_2^{\bullet-}$) (78). Although mutation of *mtsABC* does not affect transcription of *sodA*, the *mtsABC* mutant strain has decreased SOD activity and an increased sensitivity to $O_2^{\bullet-}$. Resistance to $O_2^{\bullet-}$ can be restored to the *mtsABC* strain by supplementing the medium with Mn(II), suggesting that the reduced SOD activity is due to the lack of the Mn(II) cofactor (44).

The role of metal ion homeostasis systems in protection against ROS has also been studied in related Streptococci,

including *Streptococcus pneumoniae* and *S. mutans*. *S. pneumoniae* is notable for its ability to produce millimolar levels of H_2O_2 during aerobic growth. In contrast with *E. coli* and most other systems studied to date, H_2O_2-mediated killing is unaffected by iron chelators and appears to be independent of Fenton chemistry. This is despite the fact that intracellular levels of chelatable iron and rates of hydroxyl radical production are comparable between *E. coli* and *S. pneumoniae* (74). It is suggested that perhaps Dpr prevents the association of redox active Fe(II) with DNA. In *S. mutans*, Dpr has also been described as a key factor in tolerance to oxygen due to its ability to sequester iron and prevent Fenton chemistry (99, 100). Studies in the Streptococci are generally consistent with a model in which iron homeostasis is critical to avoid ROS toxicity, Mn(II) import is important for maintaining SOD in an active form, and metal ion efflux (mediated by PmtA) contributes to resistance. However, the precise details and the apparent differences between these systems are not yet well understood.

Bradyrhizobium japonicum

B. japonicum is a soil bacterium that establishes a symbiotic relationship with host plants such as soybean in which it forms nitrogen fixing nodules associated with the plant roots. *B. japonicum* has a robust defense against ROS, but this differs in several ways from *E. coli*. Specifically, *B. japonicum* has only one primary catalase, KatG, and a *katG* mutant grows very poorly in aerobic conditions (69). The genome of *B. japonicum* encodes additional catalases/peroxidases, including *ahpC*, but they do not appear to have significant activity when cells are grown in nonstressed conditions (69). In contrast, in *E. coli*
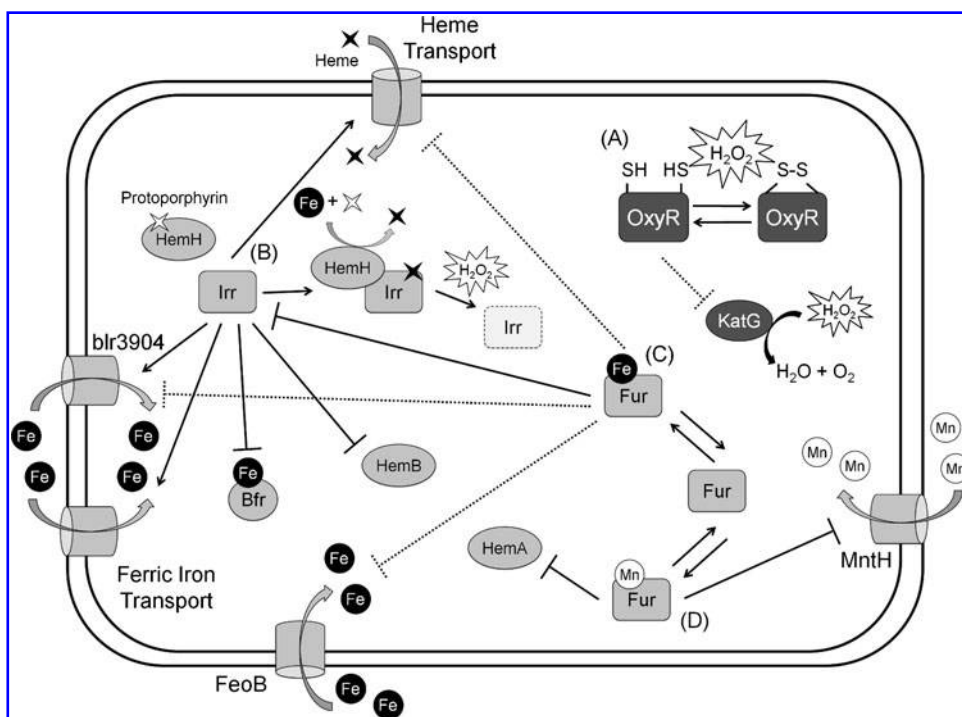
AhpCF is considered the primary enzyme responsible for protection against endogenously produced peroxide (83). Although *B. japonicum* encodes an OxyR homolog, the role of this regulator is not yet clear and an *oxyR* mutant has a similar resistance to H_2O_2 as wildtype (69).

The primary regulator responsible for controlling the response to oxidative stress and metal homeostasis in *B. japonicum* is a unique member of the Fur family, the iron response regulator (Irr) (85). Irr regulates intracellular heme levels through partially controlling heme biosynthesis and the import of heme from the extracellular environment (Fig. 7). In addition, Irr regulates iron uptake and storage. For example, under iron-limiting conditions Irr represses the transcription of a putative bacterioferritin (81). Unlike other members of the Fur family, Irr is active in its un-metallated form. Indeed, Irr is degraded in high iron conditions in response to direct binding of heme to Irr mediated by ferrochelatase, the enzyme responsible for the insertion of iron into protoporphyrin. In conditions of low iron, protoporphyrin binds to ferrochelatase, Irr and ferrochelatase do not form a complex, and Irr is free and active. Thus, Irr responds to heme levels directly at the site of heme biosynthesis. The heme-dependent degradation of Irr requires oxygen and thus also serves to sense ROS; mutation of *katG* or exposure of the cells to exogenous H_2O_2 promotes Irr degradation. *In vitro*, Irr becomes oxidized (carbonylated) in a heme-dependent manner (101). Irr thus coordinates iron homeostasis and heme biosynthesis in response to both iron availability (as sensed through heme) and oxidative stress. Phylogenomic studies indicate that this type of regulatory circuitry is likely to be widespread within the alpha-proteobacteria (45).

In addition to Irr, *B. japonicum* expresses Fur, but this protein plays a relatively minor role in iron homeostasis. In fact, this protein responds to both Fe(II) and Mn(II), whereas, in related members of the Rhizobia, the Fur homolog functions as an Mn(II) uptake regulator designated Mur. *B. japonicum* Fur is similar to PerR in that it appears to integrate information about the relative levels of Fe(II) and Mn(II). For example, Fur regulates *hemA*, encoding ALA synthase responsible for the first step of heme biosynthesis. Unlike most Fur-regulated genes, *hemA* is expressed in Fe-replete conditions in wildtype, but is constitutive and unresponsive to iron in a *fur* mutant (31). One possible explanation for this Fe-dependent regulation of *hemA* is that Mn-bound Fur, but not Fe-bound Fur, can repress *hemA* (Fig. 7). Fur can thereby regulate heme biosynthesis and uptake in response to the Mn:Fe ratio. In contrast with *hemA*, *hemB* is regulated instead by Irr. Fur also regulates, in response to Mn(II), expression of *mntH* encoding the major Mn transport protein in *B. japonicum* (34).

Fur and Irr together regulate the transcription of *irr* in a manner responsive to the Mn:Fe ratio (76). In low iron, Irr acts as an anti-repressor to Fur (presumably the Mn-bound form) on the *irr* promoter. This antirepression is particularly noticeable in low iron, but high manganese, conditions (35). This dual regulation serves to coordinate Mn and Fe levels, reducing Fe levels when Mn levels are low (76). Since the only genes shown thus far to be directly regulated by Fur are *irr* and *mntH*, it remains possible that Fur is functioning primarily as an Mn(II) sensor (Mur) and the effects on iron and heme transport in a *fur* mutant strain result from the regulation of Irr by Fur (Fig. 7).

FIG. 7. The Irr and Fur regulons of *Bradyrhizobium japonicum*. (A) OxyR does not appear to play a large role in the regulation of gene expression in *B. japonicum*. The oxidation of OxyR appears to upregulate the expression of catalase; however, this effect may be indirect, and appears to result from the derepression of *katG* by reduced OxyR. **(B)** Irr appears to play an important role in the coordination of the oxidative stress response with iron and heme homeostasis. In Fe-limited conditions, Irr is active as a regulatory protein. However, when Fe and heme are plentiful, Irr is degraded in a peroxide dependent manner. Irr is responsible for the regulation of heme transport and heme biosynthesis, inversely regulating import with synthesis. Irr also regulates Fe import and Fe storage. **(C)** In addition to heme directly regulating Irr activity, Fur:Fe represses the transcription of *irr*, preventing its production in conditions where Irr would be degraded. Fur may also regulate heme and iron homeostasis; however, these effects by Fur:Fe have not yet been shown to be direct **(D)**. In addition to Fe, Fur also binds Mn. Fur:Mn appears to regulate Mn import and possibly heme biosynthesis. Direct effects are shown as *solid lines* and potentially indirect effects are shown as *dotted lines*.

Conclusions and Perspective

Each of the six bacterial systems reviewed here provides a unique perspective on how cells adapt to peroxide stress by modulation of metal ion homeostasis. *E. coli* and the OxyR inducible stress response has provided a useful model for many Gram-negative bacteria, whereas *B. subtilis* and the PerR regulon has served this purpose for many Gram-positive organisms. These regulators are distinct as OxyR senses H_2O_2 *via* cysteine oxidation, whereas PerR relies on iron-catalyzed protein oxidation.

One of the more ubiquitous adaptations and one shared by both of these model systems is the peroxide-induction of an Fe(II) sequestering protein of the Dps family. A key role for Dps (and related proteins) in mediating peroxide resistance has now been documented in many systems, but important questions remain. It is not clear why many bacteria encode multiple Dps paralogs, and the division of labor between Dps mini-ferritins and the large ferritins is not always clear. Moreover, the fate of iron, once mineralized within Dps proteins, is not known. This iron may be irreversibly deposited as a mineral and thereby rendered inert, or it may represent a storage form that can, by processes yet to be described, be mobilized to provide iron for cellular needs. Oxidative stress may also decrease the import of iron by, for example, an OxyR-mediated increase in *fur* transcription. While oxidative stress induced efflux of iron has not been documented, this might be one function of the PmtA metal exporter described in *S. pyogenes*.

In addition to reducing the pools of reactive iron in the cell, cells also decrease the harm caused by Fenton chemistry by import of competing metals such as Mn(II) and Zn(II). Manganese, in particular, has been increasingly appreciated as a central player in the resistance to ROS and the Mn:Fe ratio within cells has emerged as a key parameter for protecting proteins against radical mediated damage. The basal levels of Mn(II) within cells varies widely. There are high Mn(II) levels in many Gram-positive bacteria and in these systems sequestration of Fe(II) may be sufficient to elevate the Mn:Fe ratio. In contrast, there is little Mn(II) in nonstressed *E. coli*, and in this case inducible import is additionally required for peroxide resistance. Numerous challenges remain, however, and the hard work of accurately measuring the physiologically relevant metal ions in these systems is in its early stages. It is very difficult to predict metal selectivity from sequence alone, and this has plagued efforts to assign function to transporters that import and export metals.

It has also proven challenging to define the precise functions of metalloregulators, and, in several cases, their perceived roles continue to evolve. For example, a putative Neisserial peroxide sensor (PerR) may in fact be more appropriately described as a Zur, and the Fur proteins of the Rhizobia have, in several cases, been re-defined as Mn(II) sensors and re-named as Mur. Indeed, in many organisms the Fur protein may be more properly considered a divalent ion sensor since both Fe(II) and Mn(II) can act as agonists to enable DNA binding. In at least some cases, the regulatory action of Fur family proteins is metal ion selective; examples include *B. subtilis* PerR and *B. japonicum* Fur, both of which appear to regulate distinct sets of genes depending on their associated regulatory metal ion. Alternatively, iron-specific sensing may be achieved by monitoring an iron-specific product such as heme (*B. japonicum* Irr) or assembly of Fe_2S_2 clusters (*E. coli* IscR). Often, the greatest insight into the regulatory logic of these and related systems emerges not from consideration of protein structure, but from a careful functional analysis of the regulated target genes. Ultimately, direct measurements of metal-protein binding interactions will be required to disentangle the complexities of the often overlapping regulatory and transport systems that control metal ion homeostasis. This work is now underway in these and related systems.

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Abbreviations Used

Bfr = bacterioferritin
Dpr = a Dps homolog
Dps = DNA-binding protein, stationary phase (mini-ferritin)
DtxR = diphtheria toxin repressor
Fur = ferric uptake regulator
Gy = absorbed radiation dose
H_2O_2 = hydrogen peroxide
Hpx = hydroperoxidases
Irr = iron responsive regulator
Isc = Fe-S cluster assembly machinery (housekeeping role)
MCO = metal-catalyzed oxidation
MntH = proton-dependent Mn(II) import
MrgA = a Dps homolog
Mur = an Mn(II)-sensing Fur homolog
OxyR = oxidative stress regulator
PerR = peroxide stress response regulator
ROS = reactive oxygen species
SOD = superoxide dismutase
Suf = a peroxide-inducible Fe-S cluster assembly system
Zur = a Zn(II)-sensing Fur homolog

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