MINIREVIEW

The Mismetallation of Enzymes during Oxidative Stress[*]

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Mononuclear iron enzymes can tightly bind non-activating metals. How do cells avoid mismetallation? The model bacterium *Escherichia coli* may control its metal pools so that thermodynamics favor the correct metallation of each enzyme. This system is disrupted, however, by superoxide and hydrogen peroxide. These species oxidize ferrous iron and thereby displace it from many iron-dependent mononuclear enzymes. Ultimately, zinc binds in its place, confers little activity, and imposes metabolic bottlenecks. Data suggest that *E. coli* compensates by using thiols to extract the zinc and by importing manganese to replace the catalytic iron atom. Manganese resists oxidants and provides substantial activity.

Ferrous iron is an excellent surface catalyst, adeptly binding and activating anionic metabolites for non-redox reactions. Because the primordial Earth was anoxic, environmental iron was present in its reduced form, which is relatively soluble (1, 2). Thus this metal was recruited to be a cofactor in numerous enzymes, and the emerging metabolic pathways were configured around the chemistry that iron can perform. That anoxic habitat persisted for at least two billion years, during which cellular biochemistry and metabolic networks became highly refined and integrated.

The subsequent appearance of photosystem II triggered a slow-moving cataclysm. Over the next 2 billion years oxygenic photosynthesis progressively filled the atmosphere with oxygen. Oxidized iron precipitated from the Earth's seas, forcing iron-dependent organisms to evolve complex strategies to obtain this necessary metal. Simultaneously, they struggled to find ways of coping with the threat of reactive oxygen species. There is a growing recognition that these problems tightly intertwine. Studies have revealed that oxidants primarily target iron enzymes and that cells adjust their metal usage to compensate. This review focuses upon these processes in *Escherichia coli*, a model organism in which knowledge of metal and stress metabolism is highly integrated.

How Do Cells Control What Metal Enters Which Enzyme?

Transition metals are often incorporated into larger cofactors such as heme, molybdopterin, cobalamin, and iron-sulfur (Fe-S) clusters. The metals bind first to assembly proteins and then are transferred to the ultimate ligands, and these two steps

provide substantial metal specificity. In contrast, most mononuclear enzymes, proteins that use polypeptide residues to bind a single metal atom, appear not to employ any protein-based metal chaperone system. Therefore the identity of the bound metal must be dictated by the intrinsic metal binding properties of the protein and the availability of metals inside the cell. These determinants of metal specificity will be explored here because oxidative stress disrupts both the metalloproteins and the metal pools.

The primary divalent transition metals in the *E. coli* cytoplasm are iron, zinc, and manganese. (Nickel is delivered by protein chaperones to a single enzyme, hydrogenase (3); copper is restricted to the extra-cytoplasmic periplasm, where all copper-dependent enzymes reside; and cobalt is not used at all.) Although Fe^{2+}, Zn^{2+}, and Mn^{2+} favor similar coordination geometries and ligands, in a given enzyme one metal may provide far more activity than another. This is obvious for redox enzymes, such as superoxide dismutase and ribonucleotide reductase, which provide ligand spheres that poise only a particular metal at the correct potential for catalysis. Mismetallation can happen *in vivo*, leading to inactive enzyme, and this observation was the first to indicate that no highly gated chaperone system controls metal access to mononuclear enzymes. It appears that some of these redox enzymes use multistep loading strategies to minimize mismetallation (4, 5).

A larger problem arises with non-redox enzymes that use divalent metals to execute surface chemistry, and these are the focus of this review. An example is ribulose-3-phosphate 5-epimerase (Rpe),[2] a member of the pentose phosphate pathway (Fig. 1). Four polypeptide residues coordinate its iron atom, but the metal remains exposed to solvent so that it can bind substrate (6). Epimerization entails deprotonation of a weakly acid carbon atom, and the key role of the Fe^{2+} is to electrostatically support this step by stabilizing the anion that is formed. Other mononuclear iron enzymes catalyze a variety of reaction classes, but they have in common the use of divalent iron to neutralize oxyanion intermediates (7–11).

One might expect that other divalent metals could also play this role, and indeed turnover numbers drop only slightly (30–50%) when manganese is substituted for iron (7). However, they plummet by up to 95% when the enzymes are charged with zinc. This poor activity may indicate that Zn^{2+} is slow to make the necessary shifts between distorted tetrahedral and octahedral geometries as substrates bind and products depart; or, because Zn^{2+} binds ligands with especially high avidity, it could reflect the sluggish release of product. In any case, the inactivity of zinc-loaded enzyme poses a real problem because these binding sites generally exhibit metal affinities in the order zinc $\gg$ iron $>$ manganese. For example, manganese dissociated from purified Rpe with a half-time of a few minutes, iron did so in 50 min, and zinc did not detectably dissociate in 8 h (12). This

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[2] The abbreviations used are: Rpe, ribulose-5-phosphate 3-epimerase; SOD, superoxide dismutase; Fur, ferric uptake regulator; DAHPS, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase; TCA, tricarboxylic acid.
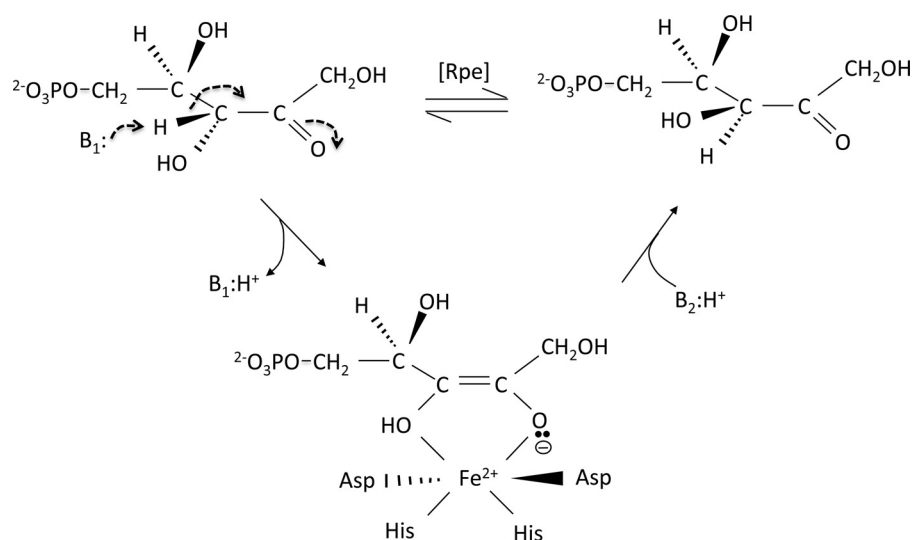
FIGURE 1. **The role of Fe^{2+} in catalysis by ribulose-5-phosphate 3-epimerase.** Proton abstraction from the chiral carbon is possible only because the divalent metal stabilizes the resonance structure of the oxyanion. Reprotonation follows, with inversion of configuration.

order mirrors the Irving-Williams series (13) and therefore is difficult for evolution to circumvent. Clearly, the cell must do something special to overcome these preferences and get the metallation process right.

The key seems to be that cells carefully control the sizes of their cytoplasmic metal pools. These pools are composed of divalent metals that weakly and reversibly associate with various metabolites, membranes, protein surfaces, and nucleic acids. Whereas the total iron content of *E. coli* is $\sim 10^{-3}$ M, most of the metal is tightly bound in dedicated enzyme sites, and the loose-iron pool is only $\sim 10^{-5}$ M (14). Total zinc (10^{-4} M) and manganese (10^{-5} to 10^{-4} M) levels are known (15, 16), but it is less clear what fraction is available to metallate apoproteins.

Metal pools arise from the balance between the activities of importer and exporter (or storage) proteins, and so the homeostatic point is set by metal-specific transcription factors that control their synthesis. The Fur transcription factor reversibly binds Fe^{2+}; in the Fur:Fe form it represses synthesis of importers and activates synthesis of ferritin, an iron-storage protein (17, 18). Manganese-loaded MntR represses synthesis of MntH, the manganese importer, and activates synthesis of MntP, an exporter (19). Zinc levels are set by two complementary regulators: Zur binds Zn^{2+} with high affinity and blocks further synthesis of the Znu importer, whereas ZntR binds Zn^{2+} with somewhat lower affinity and induces the ZntA exporter (15, 20).

This scheme implies that cytoplasmic metal levels should match the binding constants of these transcription factors. These constants have been measured but can be misconstrued. For example, ZntR exhibits a dissociation constant for zinc of 10^{-15} M (15, 21) *measured against fully hydrated zinc*. At first blush this value seems untenable because as a concentration it represents $<10^{-6}$ molecules per cell. Indeed, if the concentration of zinc available to bind nascent apoprotein were only 10^{-15} M, then even a diffusion-limited (10^{9} M^{-1} s^{-1}) binding process would require a week to activate half of an apoprotein population. One resolution of this conundrum is that virtually none of the zinc pool is fully hydrated; instead, a large (10^{-4} M)

pool of zinc is loosely associated with various biomolecules that can transfer it to zinc-requiring apoproteins via ligand exchange. *In vitro* studies support this idea (22). Another possibility is that the zinc pool is much larger than 10^{-15} M and that ZntR tracks zinc levels according to its rate of zinc acquisition, without achieving equilibrium. Indeed, data suggest that the concentration of hydrated zinc inside *E. coli* is about 10^{-11} M (23). Further, zinc-binding proteins appear to have binding constants in the 10^{-12} M range, much lower than that of ZntR (22). This disparity suggests that ZntR metallation may be kinetically determined (23), whereas much enzyme metallation is thermodynamically determined.

Although these binding constants do not directly represent the real concentrations of available metal, they do enable one to estimate the metal occupancy of proteins, if one assumes that thermodynamic equilibrium is attained. For example, if Fur ($K_D = 10^{-6}$ M (24)) establishes a loose-iron pool with a nominal concentration of 10^{-6} M hydrated iron, then at equilibrium this pool would ensure that a protein with an iron-binding site of 10^{-8} M affinity is 99% occupied. The problem becomes more interesting if one posits that this theoretical protein also has a dissociation constant for zinc of 10^{-10} M. This affinity is substantially greater than that for iron, in accordance with the behavior of most metal-binding sites. However, if the nominal availability of hydrated zinc is 10^{-11} M, zinc would populate only 10% of the enzyme population. Zinc metallation would predominate only if zinc levels rose or if iron became scarce. At the same time, an authentic zinc-dependent enzyme might have dissociation constants of 10^{-12} M for zinc and 10^{-6} for iron and would be 99% zinc-bound.

Whether this scheme correctly describes the situation for mononuclear iron enzymes depends upon their affinities for competing metals. To date a full set of binding constants has not been determined for any of these proteins, and so this model must be regarded as tentative. Still, these enzymes have exposed metal sites and exhibit iron and manganese dissociation on a minute time scale *in vitro*; similar behavior in the cell should easily allow the protein to equilibrate with the loose-

metal pool. Data described below indicate that even zinc dissociates from mononuclear iron enzymes in the relevant time frame *in vivo* (25). Thus a thermodynamic model of metal sorting is plausible for this particular class of enzymes. Note that this is less likely to be true for other groups of proteins that fold entirely around their metals, thereby prohibiting release and equilibration.

The Mechanisms of Oxidative Stress

These determinants of protein metallation turn out to be important in the context of oxidative stress. Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are continuously generated in aerated cells (26). These species are also formed by the chemical oxidation of sulfur compounds in oxic/anoxic interfaces and by the antimicrobial oxidative bursts of amoebae, plants, and mammalian phagocytes (27–29). Exogenous H_2O_2 can cross membranes into bacteria and confer stress; external O_2^-, a charged species at physiological pH ($pK_a = 4.8$), cannot (30–32).

The toxicity of these species was proven by the growth defects of scavenger-deficient mutants of *E. coli*. Strains that lack cytoplasmic superoxide dismutase (SOD$^-$) cannot catabolize TCA-cycle substrates, such as acetate, and they require supplementation with branched-chain (Ile, Leu, Val), aromatic (Phe, Trp, Tyr), and sulfur-containing (Met, Cys) amino acids (33). *E. coli* uses two catalases and an NADH peroxidase to scavenge H_2O_2, and mutants lacking these three enzymes (Hpx$^-$) exhibit defects that overlap with those of SOD$^-$ strains: they cannot catabolize TCA substrates, and they require branched-chain and aromatic supplements (34, 35). They also display a high rate of mutagenesis (36).

The mutagenicity of H_2O_2 arises from DNA damage, and the underlying chemistry involves electron transfer from ferrous iron in the Fenton reaction (37).

$$Fe^{2+} + H_2O_2 \rightarrow [FeO^{2+}] \rightarrow Fe^{3+} + OH^- + HO^\bullet$$
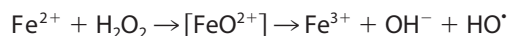
REACTION 1

Some of the cellular pool of loose iron adheres to the surface of DNA (38), and the formation of hydroxyl radicals at that site leads to DNA lesions. It is the threat of oxidative DNA damage that forces *E. coli* to limit the concentration of intracellular iron. In mutants that lack Fur protein, the loose-iron pools swell and mutation rates rise (14, 39). Under anoxic conditions, where H_2O_2 is unlikely, *E. coli* relaxes this constraint and allows more iron to enter (40).

Severe stress occurs when H_2O_2 flows into the cell from environmental sources. In this circumstance most microbes activate stress responses. The H_2O_2 directly oxidizes a sensory cysteine residue on the OxyR protein itself, creating a disulfide bond that activates it as a transcription factor (41, 42). Members of the regulon have been identified by transcriptomic analyses (43). Three of these play important roles in reducing loose-iron levels and thereby suppressing DNA damage: Dps, Fur, and YaaA. Dps is a ferritin-like protein that slows Fenton chemistry by sequestering iron (44, 45). Mutants lacking Dps suffer profuse DNA damage during protracted H_2O_2 exposure (36). The induction of Fur diminishes iron pools by repressing iron-im-

port systems (46). YaaA helps keep iron levels low as well, although the mechanism of its action is obscure (47). Collectively, these adjustments enable *E. coli* to survive encounters with physiological (micromolar) doses of H_2O_2.

Inactivation of Iron-Sulfur Clusters

However, the primary effect of oxidative stress is not to kill cells but to block growth. The TCA-cycle and branched-chain biosynthetic defects arise when O_2^- and H_2O_2 inactivate dehydratases that lie in these pathways (34, 48, 49). These enzymes are distinguished by their $[4Fe-4S]^{2+}$ clusters. Unlike electron-transfer clusters, the dehydratase clusters are exposed within the active site so they can directly bind substrate, an arrangement that leaves them accessible to dissolved oxidants. The O_2^- anion is electrostatically attracted to the cluster; presumably, it forms a complex, and if it is momentarily protonated, it exerts its oxidizing potential (+ 0.94 V) by abstracting an electron. The $[4Fe-4S]^{3+}$ species that is thereby formed is unstable and releases an iron atom into the bulk solution. A $[3Fe-4S]^+$ species is left behind. In this form the enzyme is inactive, and the pathway that it belongs to cannot operate. The TCA cycle employs two such enzymes, aconitase and fumarase, whereas the branched-chain biosynthetic pathways include dihydroxy-acid dehydratase and isopropylmalate isomerase (48, 50–52).

Hydrogen peroxide can attack the same clusters in a reaction analogous to the Fenton reaction (34). Although one might expect a hydroxyl radical to be formed, evidence indicates that the transfer of a second electron from the cluster preemptively quenches the nascent ferryl radical. Nevertheless, the catalytic iron atom is again lost, leading to the same metabolic blocks that result from O_2^- stress. The damaged enzyme can be repaired *in vitro* by the addition of reductant and ferrous iron. Enzyme reactivation occurs *in vivo* as well, with a half-time of 5–10 min (53). No particular proteins have been shown to be involved in repair of $[3Fe-4S]$ clusters, and it is plausible that the process is solely chemical, with electron donation from reductants such as cysteine and metallation by the passive binding of Fe^{2+}. Other metals apparently do not bind to the $[3Fe-4S]$ form, so this class of enzyme is protected from mismetallation.

Damage to Mononuclear Iron Enzymes

The elucidation of the TCA and branched-chain phenotypes laid the foundation for understanding the aromatic biosynthetic defect of Hpx$^-$ and SOD$^-$ strains. In principle, the problem might arise from a block in either the aromatic pathway itself or the pentose phosphate pathway, which generates erythrose-4-phosphate as an aromatic precursor. Neither pathway employs Fe-S enzymes. Metabolic analysis ultimately showed that both pathways suffer bottlenecks. As little as 0.5 μM cytoplasmic H_2O_2 specifically inactivates Rpe of the pentose phosphate pathway (12) and 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHPS) of the aromatic pathway (35). Both enzymes use Fe^{2+} to directly bind substrate. The concurrence of these two examples suggested that non-redox mononuclear iron enzymes as a class might be highly sensitive targets of H_2O_2. This prediction was validated through analysis of three additional *E. coli* enzymes, peptide deformylase, cytosine deaminase, and threonine dehydrogenase, all of which were

inactivated by low-grade H_2O_2 stress both *in vitro* and *in vivo* (7). It is notable that although the five enzymes all catalyze different types of reactions, they each use lone Fe^{2+} atoms to bind substrate and to electrostatically stabilize an oxyanionic intermediate. This action requires that the iron atom be solvent-exposed; like the catalytic iron atom in [4Fe-4S] dehydratases, this exposure allows access to small oxidants. Oxidation by either H_2O_2 or O_2^- converts the iron to its ferric form, which dissociates, leaving behind an inactive apoprotein. When this oxidation occurs *in vitro*, the enzymes can be reactivated by the addition of Fe^{2+}.

The literature on these enzymes was long unclear as to which metal serves as cofactor. The ambiguity arose from the quick dissociation of metals during purification, plus the fact that enzyme activity is not necessarily greatest with the native metal. Rpe, for example, exhibits maximal activity in the order Co(II) > Fe(II) > Mn(II) ≫ Zn(II) (12). The literature on DAHPS is particularly lively, with various authors proposing at one point or another that the physiological metal was cobalt, copper, manganese, iron, or zinc (54–59). However, only the iron-charged enzyme is sensitive to H_2O_2 *in vitro*, and the recognition that H_2O_2 poisons the enzyme *in vivo* proved that iron is its native metal. One wonders, then, how many non-redox enzymes that are annotated as using single manganese, zinc, or cobalt atoms are actually cofactored by iron *in vivo*.

Measurements revealed that O_2^- inactivates mononuclear enzymes with the same rate constant ($1–5 \times 10^6$ $\text{M}^{-1}\,\text{s}^{-1}$) with which it inactivates [4Fe-4S] dehydratases (25, 49). This makes sense because in both cases the rate-limiting step is likely to be complexation of the exposed catalytic iron atom. Given the estimated concentration of O_2^- in wild-type cells (10^{-10} M) (26), the upshot is that these enzymes will be inactivated by endogenous O_2^- every 30 min or so. Repair processes countervail this effect, so that at any moment the majority of the enzymes are functional (26). However, there is a striking discrepancy in the timing of the auxotrophies that O_2^- confers. If branched-chain amino acids are not provided, an SOD$^-$ strain stops growing almost immediately upon aeration. In contrast, the aromatic defect requires hours to be fully manifest (Fig. 2). What is the source of this difference?

Assays revealed that in SOD$^-$ mutants the activities of dehydratases declined within minutes, whereas the mononuclear enzymes retained substantial activity until much later (12, 35, 53). Importantly, although simple Fe^{2+} addition fully reactivated mononuclear enzymes that had been oxidized by O_2^- *in vitro*, it failed to restore any activity to extracts from SOD$^-$ cells. The scant residual activity of the extracts was fully resistant to H_2O_2, indicating that it originated from a metal other than iron. The K_m value of the activity matched that of zinc-loaded enzymes, and sequential treatment with penicillamine, which can extract zinc, and then iron allowed full activity to be restored. This pattern was observed for DAHPS, Rpe, and threonine dehydrogenase.

Thus the current model (Fig. 3) is that oxidation *in vivo* starts as it does *in vitro*: O_2^- oxidizes the ferrous iron and triggers its dissociation. However, in the cellular environment a pool of Fe^{2+} is immediately available to bind apoproteins, probably within seconds; thus the steady-state activity, which represents
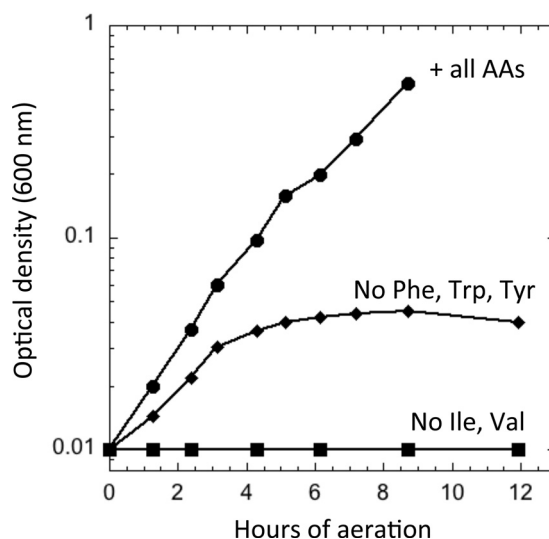
FIGURE 2. **Immediate and delayed phenotypes of superoxide stress in *E. coli*.** A SOD$^-$ mutant growing in anoxic medium was shifted at time 0 into oxic conditions. When Ile and Val were absent, growth stopped immediately due to the inactivation of dihydroxyacid dehydratase, a [4Fe-4S] enzyme. In contrast, growth continued for several hours in the aromatic-deficient medium. Enzyme analysis showed that mononuclear iron enzymes lost activity only slowly. At time 0 these enzymes were populated by ferrous iron; at 6 h they were populated by zinc. *all AAs*, all amino acids.
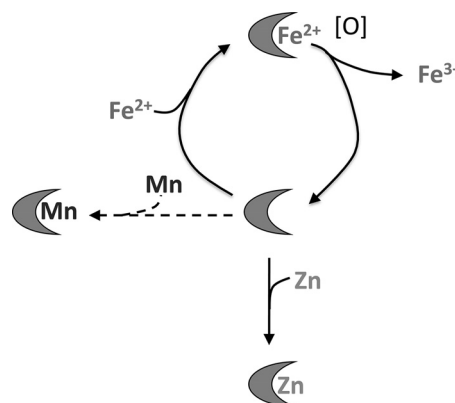
FIGURE 3. **Model for metallation of mononuclear iron enzymes during oxidative stress.** Superoxide oxidizes bound ferrous cofactor to Fe^{3+}, which dissociates, leaving behind an inactive apoprotein. Remetallation by cellular Fe^{2+} is rapid, and steady-state activity initially remains high. However, with each cycle of demetallation a subfraction of enzyme is inappropriately metallated by Zn^{2+}. Because zinc-cofactored enzyme has minimal activity, the pool of enzyme activity progressively declines, and metabolic bottlenecks ensue. Supplementation with Mn^{2+} restores enzyme activity and pathway functions, presumably due to the alternative metallation of these enzymes by manganese.

the dynamic equilibrium between oxidation and remetallation, remains high. In contrast, the repair of damaged [4Fe-4S] dehydratases is far slower, so their net activities quickly decline. However, in stressed cells the cycle of mononuclear-enzyme demetallation and remetallation repeats itself continuously, and with each iteration there is a finite chance that zinc will bind to the apoprotein in place of iron. When it finally does, it sticks tightly in the active site, and because the zinc-loaded enzyme possesses little activity, a pathway bottleneck develops.

This model implies that iron and zinc routinely compete to metallate apoproteins, which is a tenet of the thermodynamic metal-loading model. In fact, when $\Delta zntA$ mutants that cannot
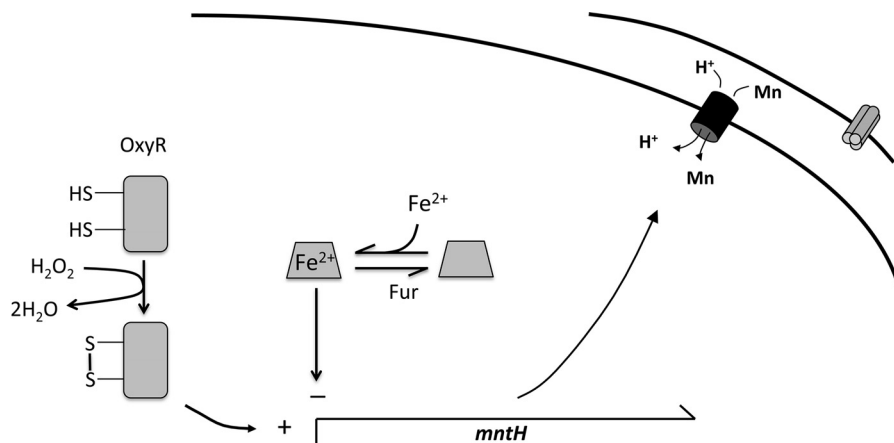
FIGURE 4. **Primary controls upon synthesis of the *E. coli* manganese importer.** The *mntH* gene is repressed in unstressed, iron-sufficient cells. When iron levels fall, the Fur repressor is inactivated by demetallation, and transcription is induced. Transcription is also induced when H_2O_2 activates the OxyR transcription factor. Under both conditions, intracellular manganese levels rise and mononuclear enzymes retain function.

export zinc were grown in zinc-rich medium, they became zinc-overloaded and exhibited a similar mismetallation of mononuclear iron enzymes (25). Thus the basic competition between iron and zinc for these enzymes was affirmed. The competition is simply exacerbated by oxidants that repeatedly displace bound iron.

Do Cells Rescue Mismetallated Enzymes?

The thermodynamic model also requires that metal binding be reversible so that an equilibrium can be achieved. Superoxide stress was used to test that idea. When SOD^- mutants were returned to an anoxic habitat, O_2^- stress ended, and over the succeeding 30 min, the activities of zinc-loaded mononuclear iron enzymes rose back to wild-type levels, although new protein synthesis was blocked (25). Examination confirmed that these enzymes had exchanged zinc for iron. Spontaneous zinc dissociation requires days *in vitro*, so how does it happen so quickly *in vivo*?

Penicillamine, a cysteine analogue, can extract zinc from active sites, suggesting that cysteine might be able to do the same. *In vitro* experiments confirmed that physiological levels of cysteine (0.2 mM) can do so on the appropriate time frame (25). Interestingly, glutathione was ineffective. Cysteine binds metals in bidentate fashion using both its sulfhydryl and primary amine moieties; in glutathione the pertinent amine is derivatized, and so its ability to coordinate metals is suppressed. The important point, however, is that these experiments demonstrate not only that iron and zinc compete for mononuclear metal sites, but that dissociation happens on a time scale that enables the process to run toward equilibrium. At least in principle, no special machinery must be invoked to favor iron binding over zinc binding.

The Protective Effect of Manganese

The role of manganese in *E. coli* metabolism is intriguing. The bacterium has a single dedicated importer, MntH, that is minimally expressed in standard laboratory media (16). Total intracellular manganese levels are quite low (~10 μM); for example, only a small fraction of the manganese-dependent superoxide dismutase is actually active (16, 60). Accordingly,

$\Delta mntH$ null mutants grow as well as wild-type strains. Thus manganese seems to have little role under typical growth conditions.

However, the *mntH* gene is controlled by Fur and is strongly induced upon iron depletion (61, 62) (Fig. 4). Iron-import-deficient strains cannot grow without it, confirming that during iron starvation manganese assumes an important role (63). Manganese-dependent SOD, NrdEF ribonucleotide reductase, and the heme biosynthetic enzyme coproporphyrinogen III oxidase (64) depend exclusively upon manganese, as they are redox enzymes reliant upon its reduction potential. These genes are induced during iron starvation, whereas during iron-replete growth their iron-dependent isozymes (SodB, NrdAB, HemN) suffice (65, 66). Thus the overall picture is that a primary role of manganese is to compensate when iron is unavailable. By extension, one anticipates that manganese might simultaneously substitute for iron in mononuclear non-redox enzymes, sparing residual iron to be used in Fe-S cluster and heme enzymes.

A similar substitution may occur during oxidative stress. For many years workers recognized that manganese supplements protect all sorts of microbes against the static effects of oxidants (67–72). Initially, it was proposed that this effect stems from the role of manganese in scavenging O_2^-, either by cofactoring SOD or by degrading O_2^- chemically (73, 74). However, although those mechanisms might occur, it was subsequently discovered that *E. coli* induces the MntH manganese importer when OxyR senses H_2O_2 stress (61). In fact, Hpx^- $\Delta mntH$ mutants are unable to grow aerobically, confirming that manganese import is critical (16). The effect is independent of O_2^- or H_2O_2 degradation. These observations raised the prospect that manganese might be an oxidant-resistant substitute for iron in mononuclear enzymes. Indeed, even modest manganese supplements to Hpx^- cells protect Rpe and DAHPS activities and fully suppress the blocks in the pentose-phosphate and aromatic pathways (12, 35).

The emerging model is that during H_2O_2 stress *E. coli* averts iron-focused damage by shifting from iron- to manganese-centered metabolism. The sequestration of iron by Dps and the

suppression of its import by Fur serve to diminish the pools of iron that might otherwise damage DNA through Fenton chemistry. Simultaneously, manganese import provides mononuclear enzymes with an oxidant-resistant cofactor that provides nearly as much activity as does iron. The diminution of iron pools is potentially problematic for Fe-S and heme synthesis, but this problem is averted when OxyR induces high titers of the Suf cluster assembly system (75) and of ferrochelatase (43).

The notion that manganese can activate iron proteins is not new. The iron sites of Fur and PerR proteins can be effectively metallated by manganese *in vitro* and *in vivo* (76, 77). Thus the metal-swap idea is mechanistically reasonable and physiologically appealing. Solid proof would lie in demonstrating that mononuclear enzymes recovered from H_2O_2-stressed cells contain manganese rather than iron. However, the experiment is technically challenging because of the high manganese dissociation rate.

One alternative model might be that manganese displaces iron from its incidental associations with metabolites, so that the released iron can metallate enzymes. However, this scheme requires the improbable notion that manganese outcompetes iron for a reservoir of adventitious ligands, whereas iron outcompetes manganese for the mononuclear protein sites.

What Lies Ahead

Substantial work remains. It must still be verified that the metallation status of non-redox iron enzymes passively follows their metal binding affinities and the sizes of the cellular metal pools. The full number of non-redox iron enzymes remains unknown even for *E. coli*, and so the full imprint of iron deficiency and of oxidative stress upon metabolism is uncertain.

A middle ground between iron and manganese centrism is struck by *Bradyrhizobium japonicum* and *Bacillus subtilis*, aerobes that require both metals for optimal growth. They employ transcription factors that are configured to respond differentially to iron and manganese and thereby carefully balance the two metal pools (77, 78). One presumes that these arrangements are dictated by the threat of enzyme mismetallation. The next step is to determine the tactics that higher organisms use solve the same problem, whether it be by coordinating metal pools, by replacing these enzymes with metal-free analogues, by compartmentalizing metals in organelles, by using chaperones, or by some combination of all of these.

REFERENCES

1. Anbar, A. D. (2008) Elements and evolution. *Science* **322,** 1481–1483
2. Dupont, C. L., Yang, S., Palenik, B., and Bourne, P. E. (2006) Modern proteomes contain putative imprints of ancient shifts in trace metal geochemistry. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 17822–17827
3. Farrugia, M. A., Macomber, L., and Hausinger, R. P. (2013) Biosynthesis of the urease metallocenter. *J. Biol. Chem.* **288,** 13178–13185
4. Whittaker, M. M., Lerch, T. F., Kirillova, O., Chapman, M. S., and Whittaker, J. W. (2011) Subunit dissociaton and metal binding by *Escherichia coli* apo-manganese superoxide dismutase. *Arch. Biochem. Biophys.* **505,** 213–225
5. Cotruvo, J. A., Jr., and Stubbe, J. (2012) Metallation and mismetallation of iron and manganese proteins *in vitro* and *in vivo*: the class I ribonucleotide reductases as a case study. *Metallomics* **4,** 1020–1036
6. Jelakovic, S., Kopriva, S., Süss, H.-H., and Schulz, G. E. (2003) Structure and catalytic mechanism of the cytosolic D-ribulose-5-phosphate 3-epimerase from rice. *J. Mol. Biol.* **326,** 127–135
7. Anjem, A., and Imlay, J. A. (2012) Mononuclear iron enzymes are primary targets of hydrogen peroxide stress. *J. Biol. Chem.* **287,** 15544–15556
8. Becker, A., Schlichting, I., Kabsch, W., Groche, D., Schultz, S., and Wagner, A. F. (1998) Iron center, substrate recognition and mechanism of peptide deformylase. *Nat. Struct. Biol.* **5,** 1053–1058
9. Ishikawa, K., Higashi, N., Nakamura, T., Matsuura, T., and Nakagawa, A. (2007) The first crystal structure of L-threonine dehydrogenase. *J. Mol. Biol.* **366,** 857–867
10. Ireton, G. C., McDermott, G., Black, M. E., and Stoddard, B. L. (2002) The structure of *Escherichia coli* cytosine deaminase. *J. Mol. Biol.* **315,** 687–697
11. Shumilin, I. A., Kretsinger, R. H., and Bauerle, R. H. (1999) Crystal structure of phenylalanine-regulated 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Escherichia coli*. *Structure* **7,** 865–875
12. Sobota, J. M., and Imlay, J. A. (2011) Iron enzyme ribulose-5-phosphate 3-epimerase in *Escherichia coli* is rapidly damaged by hydrogen peroxide but can be protected by manganese. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 5402–5407
13. Irving, H., and Williams, R. J. P. (1953) 637. The stability of transition-metal complexes. *J. Chem. Soc. (Resumed)* 3192–3210
14. Keyer, K., and Imlay, J. A. (1996) Superoxide accelerates DNA damage by elevating free-iron levels. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 13635–13640
15. Outten, C. E., and O'Halloran, T. V. (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* **292,** 2488–2492
16. Anjem, A., Varghese, S., and Imlay, J. A. (2009) Manganese import is a key element of the OxyR response to hydrogen peroxide in *Escherichia coli*. *Mol. Microbiol.* **72,** 844–858
17. Nandal, A., Huggins, C. C., Woodhall, M. R., McHugh, J., Rodríguez-Quiñones, F., Quail, M. A., Guest, J. R., and Andrews, S. C. (2010) Induction of the ferritin gene (*ftnA*) of *Escherichia coli* by Fe^{2+}-Fur is mediated by reversal of H-NS silencing and is RyhB independent. *Mol. Microbiol.* **75,** 637–657
18. Lee, J. W., and Helmann, J. D. (2007) Functional specialization within the Fur family of metalloregulators. *Biometals* **20,** 485–499
19. Waters, L. S., Sandoval, M., and Storz, G. (2011) The *Escherichia coli* MntR miniregulon includes genes encoding a small protein and an efflux pump required for manganese homeostasis. *J. Bacteriol.* **193,** 5887–5897
20. Brocklehurst, K. R., Hobman, J. L., Lawley, B., Blank, L., Marshall, S. J., Brown, N. L., and Morby, A. P. (1999) ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of *zntA* in *Escherichia coli*. *Mol. Microbiol.* **31,** 893–902
21. Ma, Z., Gabriel, S. E., and Helmann, J. D. (2011) Sequential binding and sensing of Zn(II) by *Bacillus subtilis* Zur. *Nucleic Acids Res.* **39,** 9130–9138
22. Maret, W., and Li, Y. (2009) Coordination dynamics of zinc in proteins. *Chem. Rev.* **109,** 4682–4707
23. Wang, D., Hosteen, O., and Fierke, C. A. (2012) ZntR-mediated transcription of *zntA* responds to nanomolar intracellular free zinc. *J. Inorg. Biochem.* **111,** 173–181
24. Mills, S. A., and Marletta, M. A. (2005) Metal binding characteristics and role of iron oxidation in the ferric uptake regulator from *Escherichia coli*. *Biochemistry* **44,** 13553–13559
25. Gu, M., and Imlay, J. A. (2013) Superoxide poisons mononuclear iron enzymes by causing mismetallation. *Mol. Microbiol.* **89,** 123–134
26. Imlay, J. A. (2013) The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat. Rev. Microbiol.* **11,** 443–454
27. Bedard, K., Lardy, B., and Krause, K.-H. (2007) NOX family NADPH oxidases: Not just in mammals. *Biochimie* **89,** 1107–1112
28. Glass, G. A., DeLisle, D. M., DeTogni, P., Gabig, T. G., Magee, B. H., Markert, M., and Babior, B. M. (1986) The respiratory burst oxidase of human neutrophils: further studies of the purified enzyme. *J. Biol. Chem.*

261, 13247–13251

29. Mehdy, M. C. (1994) Active oxygen species in plant defense against pathogens. *Plant Physiol.* **105,** 467–472

30. Seaver, L. C., and Imlay, J. A. (2001) Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli. J. Bacteriol.* **183,** 7182–7189

31. Lynch, R. E., and Fridovich, I. (1978) Permeation of the erythrocyte stroma by superoxide radical. *J. Biol. Chem.* **253,** 4697–4699

32. Korshunov, S. S., and Imlay, J. A. (2002) A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of phagocytosed bacteria. *Mol. Microbiol.* **43,** 95–106

33. Carlioz, A., and Touati, D. (1986) Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? *EMBO J.* **5,** 623–630

34. Jang, S., and Imlay, J. A. (2007) Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. *J. Biol. Chem.* **282,** 929–937

35. Sobota, J. M., Gu, M., and Imlay, J. A. (2014) Intracellular hydrogen peroxide and superoxide poison 3-deoxy-D-arabionheptulosonate 7-phosphate synthase, the first committed enzyme in the aromatic biosynthetic pathway of *Escherichia coli. J. Bacteriol.* **196,** 1980–1991

36. Park, S., You, X., and Imlay, J. A. (2005) Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx⁻ mutants of *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **102,** 9317–9322

37. Imlay, J. A., Chin, S. M., and Linn, S. (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction *in vivo* and *in vitro. Science* **240,** 640–642

38. Rai, P., Cole, T. D., Wemmer, D. E., and Linn, S. (2001) Localization of Fe^{2+} at an RTGR sequence within a DNA duplex explains preferential cleavage by Fe^{2+} and H_2O_2. *J. Mol. Biol.* **312,** 1089–1101

39. Touati, D., Jacques, M., Tardat, B., Bouchard, L., and Despied, S. (1995) Lethal oxidative damage and mutagenesis are generated by iron in Δ*fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. *J. Bacteriol.* **177,** 2305–2314

40. Keyer, K., Gort, A. S., and Imlay, J. A. (1995) Superoxide and the production of oxidative DNA damage. *J. Bacteriol.* **177,** 6782–6790

41. Aslund, F., Zheng, M., Beckwith, J., and Storz, G. (1999) Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 6161–6165

42. Choi, H., Kim, S., Mukhopadhyay, P., Cho, S., Woo, J., Storz, G., and Ryu, S. (2001) Structural basis of the redox switch in the OxyR transcription factor. *Cell* **105,** 103–113

43. Zheng, M., Wang, X., Templeton, L. J., Smulski, D. R., LaRossa, R. A., and Storz, G. (2001) DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J. Bacteriol.* **183,** 4562–4570

44. Ilari, A., Ceci, P., Ferrari, D., Rossi, G. L., and Chiancone, E. (2002) Iron incorporation into *E. coli* Dps gives rise to a ferritin-like microcrystalline core. *J. Biol. Chem.* **277,** 37619–37623

45. Chiancone, E., and Ceci, P. (2010) The multifaceted capacity of Dps proteins to combat bacterial stress conditions: detoxification of iron and hydrogen peroxide and DNA binding. *Biochim. Biophys. Acta* **1800,** 798–805

46. Varghese, S., Wu, A., Park, S., Imlay, K. R. C., and Imlay, J. A. (2007) Submicromolar hydrogen peroxide disrupts the ability of Fur protein to control free-iron levels in *Escherichia coli. Mol. Microbiol.* **64,** 822–830

47. Liu, Y., Bauer, S. C., and Imlay, J. A. (2011) The YaaA protein of the *Escherichia coli* OxyR regulon lessens hydrogen peroxide toxicity by diminishing the amount of intracellular unincorporated iron. *J. Bacteriol.* **193,** 2186–2196

48. Kuo, C. F., Mashino, T., and Fridovich, I. (1987) α,β-dihydroxyisovalerate dehydratase: a superoxide-sensitive enzyme. *J. Biol. Chem.* **262,** 4724–4727

49. Flint, D. H., Tuminello, J. F., and Emptage, M. H. (1993) The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J. Biol. Chem.* **268,** 22369–22376

50. Gardner, P. R., and Fridovich, I. (1991) Superoxide sensitivity of the *Escherichia coli* aconitase. *J. Biol. Chem.* **266,** 19328–19333

51. Liochev, S. I., and Fridovich, I. (1993) Modulation of the fumarases of *Escherichia coli* in response to oxidative stress. *Arch. Biochem. Biophys.* **301,** 379–384

52. Hentze, M. W., and Argos, P. (1991) Homology between IRE-BP, a regulatory RNA-binding protein, aconitase, and isopropylmalate isomerase. *Nucleic Acids Res.* **19,** 1739–1740

53. Djaman, O., Outten, F. W., and Imlay, J. A. (2004) Repair of oxidized iron-sulfur clusters in *Escherichia coli. J. Biol. Chem.* **279,** 44590–44599

54. Stephens, C. M., and Bauerle, R. (1991) Analysis of the metal requirement of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Escherichia coli. J. Biol. Chem.* **266,** 20810–20817

55. Staub, M., and Dénes, G. (1969) Purification and properties of the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (phenylalanine sensitive) of *Escherichia coli* K12. I. Purification of enzyme and some of its catalytic properties. *Biochim. Biophys. Acta* **178,** 588–598

56. McCandliss, R. J., and Herrmann, K. M. (1978) Iron, an essential element for biosynthesis of aromatic compounds. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 4810–4813

57. Baasov, T., and Knowles, J. R. (1989) Is the first enzyme of the shikimate pathway, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (tyrosine sensitive), a copper metalloenzyme? *J. Bacteriol.* **171,** 6155–6160

58. Ray, J. M., and Bauerle, R. (1991) Purification and properties of tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Escherichia coli. J. Bacteriol.* **173,** 1894–1901

59. Ramilo, C. A., and Evans, J. N. S. (1997) Overexpression, purification, and characterization of tyrosine-sensitive 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase from *Escherichia coli. Protein Expr. Purif.* **9,** 253–261

60. Pugh, S. Y. R., and Fridovich, I. (1985) Induction of superoxide dismutases in *Escherichia coli* B by metal chelators. *J. Bacteriol.* **162,** 196–202

61. Kehres, D. G., Janakiraman, A., Slauch, J. M., and Maguire, M. E. (2002) Regulation of *Salmonella enterica* serovar Typhimurium *mntH* transcription by H_2O_2, Fe^{2+}, and Mn^{2+}. *J. Bacteriol.* **184,** 3151–3158

62. Ikeda, J. S., Janakiraman, A., Kehres, D. G., Maguire, M. E., and Slauch, J. M. (2005) Transcriptional regulation of *citABCD* of *Salmonella enterica* serovar Typhimurium by MntR and Fur. *J. Bacteriol.* **187,** 912–922

63. Grass, G., Franke, S., Taudte, N., Nies, D. H., Kucharski, L. M., Maguire, M. E., and Rensing, C. (2005) The metal permease ZupT from *Escherichia coli* is a transporter with a broad substrate spectrum. *J. Bacteriol.* **187,** 1604–1611

64. Breckau, D., Mahlitz, E., Sauerwald, A., Layer, G., and Jahn, D. (2003) Oxygen-dependent coproporphyrinogen III oxidase (HemF) from *Escherichia coli* is stimulated by manganese. *J. Biol. Chem.* **278,** 46625–46631

65. Martin, J. E., and Imlay, J. A. (2011) The alternative aerobic ribonucleotide reductase of *Escherichia coli*, NrdEF, is a manganese-dependent enzyme that enables cell replication during periods of iron starvation. *Mol. Microbiol.* **80,** 319–334

66. Compan, I., and Touati, D. (1993) Interaction of six global transcription regulators in expression of manganese superoxide dismutase in *Escherichia coli* K-12. *J. Bacteriol.* **175,** 1687–1696

67. Daly, M. J., Gaidamakova, E. K., Matrosova, V. Y., Kiang, J. G., Fukumoto, R., Lee, D. Y., Wehr, N. B., Viteri, G. A., Berlett, B. S., and Levine, R. L. (2010) Small-molecule antioxidant proteome-shields in *Deinococcus radiodurans. PLoS ONE* **5,** e12570

68. Tseng, H. J., Srikhanta, Y., McEwan, A. G., and Jennings, M. P. (2001) Accumulation of manganese in *Neisseria gonorrhoeae* correlates with resistance to oxidative killing by superoxide anion and is independent of superoxide dismutase activity. *Mol. Microbiol.* **40,** 1175–1186

69. Seib, K. L., Tseng, H. J., McEwan, A. G., Apicella, M. A., and Jennings, M. P. (2004) Defenses against oxidative stress in *Neisseria gonorrhoeae* and *Neisseria meningitidis*: distinctive systems for different lifestyles. *J. Infect. Dis.* **190,** 136–147

70. Tseng, H. J., McEwan, A. G., Paton, J. C., and Jennings, M. P. (2002) Virulence of *Streptococcus pneumoniae*: PsaA mutants are hypersensitive to oxidative stress. *Infect. Immun.* **70,** 1635–1639

71. Chang, E. C., and Kosman, D. J. (1989) Intracellular Mn(II)-associated superoxide scavenging activity protects Cu,Zn superoxide dismutase-deficient *Saccharomyces cerevisiae* against dioxygen stress. *J. Biol. Chem.*

264, 12172–12178

72. Inaoka, T., Matsumura, Y., and Tsuchido, T. (1999) SodA and manganese are essential for resistance to oxidative stress in growing and sporulating cells of *Bacillus subtilis. J. Bacteriol.* **181,** 1939–1943

73. Archibald, F. S., and Fridovich, I. (1981) Manganese and defenses against oxygen toxicity in *Lactobacillus plantarum. J. Bacteriol.* **145,** 442–451

74. Barnese, K., Gralla, E. B., Valentine, J. S., and Cabelli, D. E. (2012) Biologically relevant mechanism for catalytic superoxide removal by simple manganese compounds. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 6892–6897

75. Outten, F. W., Djaman, O., and Storz, G. (2004) A *suf* operon requirement

for Fe-S cluster assembly during iron starvation in *Escherichia coli. Mol. Microbiol.* **52,** 861–872

76. Lee, J. W., and Helmann, J. D. (2006) The PerR transcription factor senses H_2O_2 by metal-catalyzed histidine oxidation. *Nature* **440,** 363–367

77. Ma, Z., Faulkner, M. J., and Helmann, J. D. (2012) Origins of the specificity and cross-talk in metal ion sensing by *Bacillus subtilis* Fur. *Mol. Microbiol.* **86,** 1144–1155

78. Yang, J., Sangwan, I., Lindemann, A., Hauser, F., Hennecke, H., Fischer, H. M., and O'Brian, M. R. (2006) *Bradyrhizobium japonicum* senses iron through the status of haem to regulate iron homeostasis and metabolism. *Mol. Microbiol.* **60,** 427–437