MINIREVIEW

Specificity of Metal Sensing: Iron and Manganese Homeostasis in *Bacillus subtilis*†

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Metalloregulatory proteins allow cells to sense metal ions and appropriately adjust the expression of metal uptake, storage, and efflux pathways. *Bacillus subtilis* provides a model for the coordinate regulation of iron and manganese homeostasis that involves three key regulators: Fur senses iron sufficiency, MntR senses manganese sufficiency, and PerR senses the intracellular Fe/Mn ratio. Here, I review the structural and physiological bases of selective metal perception, the effects of non-cognate metals, and mechanisms that may serve to coordinate iron and manganese homeostasis.

Manganese and Iron: Chemically Similar Elements with Distinct Roles in Cell Physiology

Manganese and iron are both predominantly present as divalent cations in the reducing environment of the cytoplasm and are maintained at overall concentrations (averaged over the cell) that can approach 0.5 μM (1–3). Much of this manganese and iron is bound by enzymes that acquire metal from a kinetically accessible (labile) pool buffered in the low μM range (4–6). These two metals are not only important from the perspective of individual microbial cells, but have a global impact due to their central roles in photosynthesis, nitrogen fixation, and other key steps in elemental cycling.

Nearly all cellular life requires significant amounts of iron. The sole documented exceptions are the lactobacilli and the spirochete *Borrelia burgdorferi*, which have little if any requirement for iron (3). Coincident with this disregard for iron, these same organisms have high requirements for manganese and have been described as manganese-centric (7). Conversely, other organisms have little demonstrable requirement for manganese, although they may use manganese when available. The best characterized is *Escherichia coli*, which has a largely iron-centric metabolism, but conditionally imports manganese in response to oxidative stress (8).

Here, I review the key factors regulating iron and Mn homeostasis in the model organism *Bacillus subtilis*. *B. subtilis* encodes three members of the Fur2 family (Fur, PerR, and Zur) and one member of the DtxR/MntR family (MntR) that regulate the import of nutrient metal ions (9–11). Structural and biochemical studies have provided insights into the mechanisms of selective activation of these repressors by their cognate metal ions (12–14). *B. subtilis* requires both iron and manganese for growth, a property shared with many pathogens for which the ability to obtain these metals from the host is a critical determinant of virulence (7, 15–17).

Metalloregulators as a Window into Cellular Physiology

Metalloregulators function as the arbiters of metal ion sufficiency and excess (6, 12, 18). For those that sense sufficiency, the affinity (measured as *Kd*) for their cognate metal(s) defines the level of metal judged to be sufficient. When free metal concentrations rise above this level, the regulator transitions from its inactive (apo-) form to its activated (holo-) form and represses expression of metal import. In *B. subtilis*, Fur serves as the primary sensor of iron sufficiency (19), and MntR monitors manganese sufficiency (11), with both Mn2+ and Fe2+ pools buffered to the low μM range (4).

The ability of metalloregulators to respond selectively to their cognate metals relies on a complex interplay of multiple factors that can be succinctly described as affinity, access, and allosteroy (5). Affinity is largely determined by the nature and geometry of the metal-binding ligands. However, in agreement with the Irving–Williams series, sensors of Mn2+ and Fe2+ typically bind Zn2+ with an affinity greater than their cognate metals. Nevertheless, Zn2+ does not elicit repression because cellular levels of free Zn2+ are maintained far below those for Fe2+ and Mn2+ (4). This highlights the second key determinant of selectivity: response to metals depends on access, and this can vary drastically between organisms. Finally, non-cognate metals may be discriminated against after binding: they may fail to elicit a genetic response due to an inability to trigger allosteroy.

Biochemical measurements can provide insights into the affinity of metalloregulators for metal ions. Direct measurements of metal binding in the absence of DNA are likely most relevant for metal-activated repressors that bind DNA subsequent to metal binding. Because of the thermodynamic coupling (coupling free energy) between metal and DNA binding, binding to DNA influences metal affinity to the same degree that metal binding influences DNA affinity (6). This is of particular relevance when considering sensors of metal excess that bind DNA as apo-proteins and dissociate upon metal binding. In this case, the functional metal sensor (DNA-bound apo-protein) will bind metal with an affinity significantly lower than measured for the apo-protein in solution.

Studies of metalloregulators, and in particular the identification of their target genes, also provide insights into metal physiology. In addition to the derepression of uptake systems, metal limitation may activate pathways to repress the synthesis of some metalloenzymes and to induce alternative enzymes to replace those whose activity becomes compromised when their metal cofactor is limiting (20). For example, when *B. subtilis* is zinc-limited, derepression of the Zur regulon leads to induction...
of an alternative, non-zinc-dependent enzyme required for folate biosynthesis (21). Indeed, sophisticated mechanisms to bypass what would otherwise be metabolic bottlenecks created by limitation for specific elements are legion (20).

**Metal Ion Speciation in the Cell**

The total metal content (quota) of the cell is distributed among ligands with a wide range of affinities and kinetic lability, from comparatively tight binding metalloproteins to both low molecular weight (LMW) and macromolecular anions. In both *E. coli* and *Bacillus anthracis*, the largest pools of protein-associated iron are in iron-superoxide dismutase (Fe-SOD) and oxidized to ferric hydroxide within ferritins and mini-ferritins (Dps family proteins) (22, 23). Once oxidized, stored iron no longer contributes to the labile iron pool. Mn-SOD may be a dominant pool of protein-bound manganese in Bacilli (23).

The labile pool can be defined as that portion of the metal quota that is available for equilibration with metalloregulators. The labile manganese pool is likely buffered as an LMW pool bound to phosphate, nucleotides, peptides, and organic acids (24, 25), including the abundant metabolite fructose 1,6-diphosphate (26). The labile iron pool is thought to be largely chelated by LMW thiols such as glutathione, with a lesser contribution from citrate and other organic acids (27).

The labile manganese and iron pools may represent a significant fraction of the total metal quota. Indeed, metallated enzymes may comprise part of the labile pool for these metals. For example, the metallation state of both Fe-SOD and Mn-SOD can be very sensitive to metal availability in the cell, with binding of the non-cognate ion typically leading to enzyme inhibition (8, 23, 28, 29). In addition, both iron and manganese present in mononuclear enzymes may dissociate readily during purification, suggestive of weak binding (30, 31). Direct measurements of Mn$^{2+}$ dissociation from the *B. anthracis* MntR ortholog reveal a half-time of 6 s (32), and exchange reactions on this time scale are likely common in the cytosol. To the extent that Mn$^{2+}$- and Fe$^{2+}$-cofactored enzymes rapidly exchange their cofactor, these ions may be considered part of the labile pool.

**B. subtilis Fur, MntR, and PerR Coordinate Iron and Manganese Homeostasis**

Fur, MntR, and PerR are the key regulators of iron and manganese homeostasis in *B. subtilis* (13). Fur senses the labile iron pool, whereas MntR monitors the free manganese pool (Fig. 1). PerR is a Fur paralog best known for its role in the regulation of peroxide stress genes. When metallated by iron, PerR is exquisitely sensitive to H$_2$O$_2$, which oxidizes either of two His ligands to 2-oxo-His (33, 34). In addition, PerR has a second function as sensor of the Fe/Mn ratio. In addition to Fur and PerR, a third Fur paralog, Zur, functions to maintain zinc homeostasis (10, 35).

**The Fur Regulon and Iron Homeostasis**

Fur is the central regulator of iron homeostasis in *B. subtilis* (9). Indeed, nearly all of the genes induced by the Fe$^{2+}$ chelator dipyridyl are equivalently derepressed in a fur mutant (19). Fur represses the synthesis of the bacillibactin siderophore together with pathways involved in iron uptake (36, 37). Fur also indirectly regulates many more genes as mediated by a small RNA (FsrA) in collaboration with FbpA, FbpB, and FbpC (putative RNA chaperones) (40). FsrA down-regu-
protein with a structural Cys4:Zn site (site 1) required for protein stability (Fig. 2). Fur contains two other metal-sensing sites per monomer (sites 2 and 3). Metal binds first at site 3 with an overall apparent affinity ($K_d$) of $\sim1 \mu M$ Fe$^{2+}$ in a coupled DNA binding assay. Adapted from Ref. 4.

lates low priority iron enzymes as part of an iron-sparing response with far-reaching impacts on central metabolism (41, 42).

The Precarious Nature of Iron-selective Recognition by Fur

Fur family proteins provide an instructive example of how cells can adapt a common protein scaffold for sensing of diverse metals including iron (Fur), zinc (Zur), manganese (Mur), and nickel (Nur) (43, 44). Fur in particular plays a critical role in the biology of many pathogens (45, 46). *B. subtilis* Fur responds with high selectivity to Fe$^{2+}$, but in other cases, metal sensing may be more promiscuous.

A model for metal recognition by *B. subtilis* Fur has been developed using homology modeling, competition metal binding, and mutational studies (4). Fur is a dimeric DNA-binding protein with a structural Cys$_4$:Zn site (site 1) required for protein stability (Fig. 2). Fur contains two other metal-sensing sites per monomer (sites 2 and 3). Metal binds first at site 3 with subsequent binding to the key allosteric site (site 2) leading to a $>100$-fold increase in DNA binding affinity. *In vitro*, $\sim1 \mu M$ free iron activates DNA binding (4).

Binding of Mn$^{2+}$ can also activate Fur with somewhat lower affinity ($K_d \sim24 \mu M$), which is above the estimated level of free Mn$^{2+}$ as normally monitored by MntR ($K_d \sim6 \mu M$). Thus, Mn$^{2+}$ does not activate Fur under most conditions. This contrasts with enteric bacteria where selection for manganese resistance leads to recovery of mutations in fur (47). This might suggest that manganese toxicity arises from inappropriate repression of iron import. In *Salmonella enterica*, the mntH gene is repressed by both MntR (in response to manganese) and Fur (in response to iron), but both metalloregulators can also mediate repression (albeit less efficiently) in response to the non-cognate metal (48).

Although *B. subtilis* Fur is highly specific for Fe$^{2+}$ under most conditions, cross-talk may sometimes occur. For example, the Fur regulon is moderately repressed by high levels of Cd$^{2+}$ and Zn$^{2+}$ (49). Furthermore, when Fur protein levels are elevated $\sim2$-fold (by mutation of the PerR repressor) Fur is constitutively activated by ambient Mn$^{2+}$, leading to a severe iron starvation phenotype (4, 50). This suggests that this system is delicately poised, and conditions that transiently elevate the level of either Fur protein or Mn$^{2+}$ will, by mass action, lead to manganese-cofactored Fur and repression of iron import. Cross-talk between manganese and iron clearly occurs in *E. coli* (48), but this may be less of an issue for this iron-centric bacterium because ambient manganese is generally maintained at very low levels.

The MntR Regulon and Manganese Homeostasis

MntR is a Mn$^{2+}$-specific metalloregulator structurally related to DtxR/IdeR Fe$^{2+}$ sensors (11, 51, 52). Members of this large and diverse protein family function to sense iron, manganese, or both, and are key regulators in several pathogens (53). *B. subtilis* MntR represses two manganese uptake systems: the ATP-dependent MntABCD transporter and MntH, a proton-coupled symporter (54). An mntR mutant is extremely sensitive to manganese, largely due to an inability to repress MntH (11). Unlike wild type, which tolerates 1 mM Mn$^{2+}$, an mntR mutant is unable to grow with 5 $\mu M$ Mn$^{2+}$. Thus, the ability to repress Mn$^{2+}$ uptake is critical for cell physiology.

Selective Recognition of Manganese by MntR

From a structural perspective, *B. subtilis* MntR is one of the most thoroughly studied metalloregulators and provides an interesting comparison with the iron-sensing DtxR/IdeR homologs (51) (Fig. 3). MntR binds Mn$^{2+}$ at two sites per monomer, A and C (55). Formation of this metal cluster orients the two DNA-binding recognition helices at the appropriate distance for binding to operator DNA (55, 56). Although various Mn$^{2+}$ binding affinities have been measured for MntR, recent studies reveal an overall $K_d$ of $\sim6 \mu M$ (4). Cd$^{2+}$ can also bind to MntR to repress the MntR regulon (49, 57). Although Cd$^{2+}$ is considered a non-cognate metal with respect to Mn$^{2+}$ homeostasis, Cd$^{2+}$ may be a physiologically relevant agonist; MntH is a major route of entry for the toxic cadmium ion (11).

Numerous structures are available for *B. subtilis* MntR as an apo-protein (56), in various metallated states (51, 55, 57), and for mutant proteins altered in metal recognition (57). Collectively, these structures lead to a model for manganese-selective metallo sensing in which manganese binds first to the A site with heptacoordinate ligation, and this organizes the C site to enable binding of a second manganese and stabilization of the active repressor conformation (Fig. 3C). MntR also binds tightly to non-cognate metals (Fe$^{2+}$, Co$^{2+}$, and Zn$^{2+}$), but these fail to allosterically activate DNA binding due to an inability to form a binuclear cluster (57). Fe$^{2+}$ binds at the A site but with a different geometry than manganese, and this distorts the C site such that the second metal binding event (required for DNA binding) is inhibited (57). As a result, Fe$^{2+}$ is an antagonist for MntR (11, 52, 54). Indeed, an mntR-regulated gene is not repressed even when *fur* mutant cells, derepressed for iron import, are grown in high iron conditions (52). In contrast, in *S. enterica*, MntR can respond to both Mn$^{2+}$ and Fe$^{2+}$ (48),...
although the structural differences that allow this wider effector response are not understood.

Insights into the origins of metal specificity have been obtained from structural comparisons of manganese-specific representatives such as *B. subtilis* MntR and *Streptococcus gordonii* ScaR with iron-specific family members such as Corynebacterium diphtheriae DtxR (52, 57–59). Comparison of MntR and DtxR/IdeR proteins revealed two notable differences in the first coordination sphere for the activating metal (Fig. 3B). Specifically, Met-10 in DtxR/IdeR is replaced by Asp-8 in MntR, and Cys-102 is replaced by Glu-99 (52). This suggests that sulfur-containing ligands tip the balance in favor of iron activation by altering the affinity of the metalloregulator for its activating metal(s). Studies with mutant versions of both DtxR and MntR provide partial support for this notion. For example, a D8M mutant of MntR responds to both iron and manganese in *B. subtilis*, suggesting that the presence of a thioether ligand (Met) in site C is sufficient to allow activation by iron (52).

To explore the biochemical determinants for the high iron selectivity of DtxR, this metalloregulator was expressed in *B. subtilis*. Unexpectedly, DtxR responded to both iron and manganese with comparable sensitivity in *B. subtilis* (52). This suggests that the failure of DtxR to respond to manganese in its native environment is due to a lack of access: ambient levels of manganese that are maintained (presumably by the *C. diphtheriae* ortholog of MntR (60)) at levels below those needed for activation. If this model is correct, *C. diphtheriae* MntR should bind Mn$^{2+}$ with substantially higher affinity than *B. subtilis* MntR. DtxR could be converted to a Mn$^{2+}$-selective repressor by replacement of two ligands corresponding to the MntR C site; the DtxR M10D/C102E double mutant responds to manganese but not to iron in *B. subtilis*. However, a robust response to iron is recovered in a fur mutant strain derepressed for iron import, indicating that these two amino acid substitutions have reduced, but not eliminated, the ability of iron to activate DNA binding (52). Thus, metalloregulators are finely tuned to function within their specific host milieu and, not surprisingly, evolution has optimized selectivity only to the point where a sufficiently selective response is obtained under ambient metal concentrations (5).

Collectively, these results suggest that formation of a binuclear cluster enables selective Mn$^{2+}$ sensing in MntR. However, some MntR orthologs can selectively sense Mn$^{2+}$ with a single binding site. This is the case for *S. gordonii* ScaR (58) and *Streptococcus pneumoniae* PsaR (59), which have a Lys residue positioned to replace the need for metal binding in the A site (Fig. 3D), as visualized in the structure of a manganese-bound EllK variant of *B. subtilis* MntR (57).

**PerR and the PerR Regulon**

*B. subtilis* PerR is a member of the Fur family of proteins and plays a central role in regulation of the adaptive response to H$_2$O$_2$ (61–63). Like its two paralogs (Fur and Zur), it forms a stable dimer containing one structural Zn$^{2+}$ per monomer (in a Cys$_4$ site) (64). Binding of one additional metal ion per monomer (in a site homologous to site 2 of Fur) activates DNA bind-
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FIGURE 4. Structural insights into iron and manganese recognition by B. subtilis PerR. A, superposition of the global dimer structures of the apo-repressor (PerR:Zn; brown) and holo-repressor (PerR:Zn,Mn; green). The structural zinc ions are in black, and the regulatory manganese ions are in yellow (adapted from Ref. 66). B, close-up of the PerR regulatory site with His-37, His-91, His-93, Asp-85, and Asp-104 coordinating manganese (yellow). C, close-up of the regulatory metal-binding site (metal in blue) and relationship to putative site 3 residues (Glu-114 and His-128) in PerR:Zn,Mn (PDB entry 3F8N; adapted from Ref. 93). Coordination of His-37 from the DNA-binding domain, to the bound metal ion, triggers the allosteric reorientation of the two DNA-binding domains. Oxidation of this same residue by H$_2$O$_2$ leads to derepression of the PerR regulon. D, top view of the regulatory site highlighting (in red) the hydrophilic environment that facilitates metal exchange and reaction of bound iron with H$_2$O$_2$ (adapted from Ref. 66). Note that the size of the Asp-104 side chain affects the size of the hydrophilic channel and thereby influences H$_2$O$_2$ reactivity. A D104E mutant of PerR reduces H$_2$O$_2$ sensitivity, and a reverse mutation in Fur (E108D) increases H$_2$O$_2$ reactivity (70).

B. subtilis PerR regulates peroxide defense functions including the major vegetative catalase (KatA), heme biosynthesis, alkyl hydroperoxide reductase (AhpCF), and MrgA, a homolog of the E. coli DNA-binding and iron sequestration protein, Dps (9, 38, 67). It is notable that several PerR-regulated genes (MrgA, Fur, and ZosA) have roles in metal ion homeostasis. Indeed, peroxide stress commonly elicits adaptive changes in metal homeostasis that conspire to reduce the largely iron-dependent toxicity (68).

Most studies to date of the PerR regulon have focused on the mrgA gene as a model. Repression of mrgA requires PerR and a divalent cation as a corepressor (33, 69). Both Mn$^{2+}$ and Fe$^{2+}$ activate PerR to bind DNA with high affinity, thereby allowing repression of the PerR regulon. Conversely, resuspension of growing cells in minimal medium lacking both manganese and iron leads to rapid derepression of the PerR regulon, whereas omission of either metal alone does not (69).

Under most growth conditions, PerR is predominantly in the Fe$^{2+}$ form (designated PerR:Zn,Fe). Indeed, in vitro, DNA binding is activated by Fe$^{2+}$ ($K_a = 0.1$ $\mu$M) at lower concentrations than Mn$^{2+}$ ($K_a = 2.8$ $\mu$M) (64), and when both metals are present at equivalent concentrations, the iron form predominates (33). However, in medium supplemented with manganese, and deficient for iron, PerR is in the PerR:Zn,Mn form and is insensitive to H$_2$O$_2$ (67–69). It is not entirely clear why some Fur family members, such as PerR, are highly sensitive to H$_2$O$_2$, whereas others are not, even when bound with iron. One factor that influences the H$_2$O$_2$ reactivity of bound iron may be the solvent accessibility (Fig. 4D), as inferred from the effects of amino acid substitutions in PerR and Fur (70).

Importantly, not all PerR-regulated genes respond the same to metal ion availability (69). Although manganese leads to strong repression of all PerR-regulated genes, iron only represses a subset including those functions most critical under conditions of peroxide stress such as katA, ahpCF, and mrgA. Other PerR-regulated genes, including fur and perR itself, are repressed by PerR:Zn,Mn, but not by PerR:Zn,Fe (69). For these genes, PerR does not function as a peroxide sensor (and these genes are not induced by H$_2$O$_2$), but instead acts as a sensor of the Fe/Mn ratio. The molecular basis for this differential regulation is an active area of research.

Possible Mechanisms for Coordinating Iron and Manganese Homeostasis

The high selectivity of the Fur and MntR metalloregulators, as well as the minimal cross-talk observed between the iron and manganese starvation responses, has led to a general model in which iron and manganese homeostasis are largely independent (Fig. 1). However, several of the observations summarized above suggest that they may in fact be more closely integrated than heretofore appreciated.

In addition to regulating manganese uptake via MntR, manganese may, at least under some conditions, also regulate iron homeostasis. In vitro, Mn$^{2+}$ can activate B. subtilis Fur to bind DNA, and recent results reveal that ambient Mn$^{2+}$ levels are sufficient to support constitutive (and inappropriate) repression of iron uptake when Fur protein levels are elevated from ~10,000 to ~22,000 monomers per cell due to loss of PerR repression (50). This cross-talk likely reflects the fact that the affinity of Fur for Mn$^{2+}$ ($K_a$~24 $\mu$M) is only modestly less than...
the MntR affinity for Mn\(^{2+}\) \((K_{d} \sim 6 \ \mu\text{M})\) that presumably governs intracellular manganese levels. It is possible that similar mass action effects are normally at play to fine-tune iron responsiveness in the cell. Specifically, when the Fe/Mn ratio is high (as sensed by PerR), fur will be derepressed, and this higher level of Fur protein may serve to decrease the level of iron needed to repress the Fur regulon. Conversely, when manganese is abundant, fur transcription will be reduced by PerR: Zn,Mn and higher levels of iron will be needed for the efficient repression of iron uptake. A similar coordination is apparent in the α-proteobacterium Bradyrhizobium jiponicum; manganese limitation directly regulates iron homeostasis by antagonizing the heme-dependent degradation of the key iron regulator Irr (71).

There are also several plausible mechanisms by which iron might modulate manganese homeostasis. For example, Fe\(^{2+}\) serves as an antagonist of MntR-dependent repression in vitro. Thus, under conditions of transient iron overload (such as may occur upon shift of iron-deficient cells to iron-sufficient medium or as a consequence of oxidative damage to iron-containing enzymes \((72–74)\)), iron may compete for the MntR A site and thereby impede formation of the functional repressor. One consequence would be to increase manganese uptake under conditions of iron excess, a plausible mechanism to ensure a balance between these two competitive metal ions. It has also been observed that a fur mutant strain displays down-regulation of the two major Mn\(^{2+}\) uptake systems \((mntH\text{ and } mntABCD)\), and this effect required components of the iron-sparing response \((\text{the FbpAB proteins and FsrA})\) \((41, 42)\). This raises the possibility that conditions of iron deprivation lead to both an induction of iron uptake and a concomitant down-regulation of manganese uptake. Such mechanisms may represent a strategy to balance the Fe/Mn ratio.

**Physiological Consequences of Imbalances in the Fe/Mn Ratio**

Bacteria have evolved a wide variety of mechanisms to ensure that iron and manganese levels are properly regulated, including the recent discovery of manganese efflux systems \((71, 75)\). In *E. coli*, metabolism seems to be relatively iron-centric with only conditionally useful of manganese \((8)\). Indeed, Mn-SOD, a representative manganese-dependent enzyme, is largely unmetallated in unstressed cells \((8)\). In response to oxidative stress, manganese import is derepressed, Mn-SOD acquires its cofactor, and several iron-containing enzymes switch to a less redox-sensitive manganese form \((31)\).

Unlike *E. coli*, *B. subtilis* is reliant on manganese for growth and normally maintains comparable pools of both total and labile iron and manganese.

The complexity of the interplay between iron and manganese has been explored from several directions. For example, most manganese- and iron-cofactored SODs are active only with their cognate metal due to ligand-mediated tuning of the metal redox potential \((76)\). Proper metallation of SODs appears to be particularly sensitive to metal availability \((29, 77)\). Interestingly, heterologous expression studies with Mn-SOD from the manganese-centric *B. burgdorferi* indicate that it is only metallated when the cytosol contains high levels of Mn\(^{2+}\), consistent with the ambient conditions in its native host \((28)\).

Other enzymes may also vary between Fe\(^{2+}\)- and Mn\(^{2+}\)-cofactored forms, as shown for *E. coli* peptide deformylase, threonine dehydrogenase, cytosine deaminase, and 3-deoxy-d-arabinoheptulosonate 7-phosphate synthase \((31)\). It is notable that some essential mononuclear enzymes in *B. subtilis*, including peptide deformylase and methionine aminopeptidase, have multiple isoforms with apparently distinct metal selectivity \((78, 79)\). The cellular Fe/Mn ratio has also emerged as a critical determinant of radioresistance \((1)\), and the LMW pool of labile manganese is thought to have significant protective effects under oxidative stress conditions \((25)\).

One important, but still poorly understood, area of physiology relates to the consequences of metal imbalances. When metal ions are deficient, cells will cease growth, but the nature of the resulting defects are generally not known. Some insights into the processes that fail when metals are limited can be gleaned from genes induced in response to metal starvation. For example, derepression of a manganese-cofactored ribonucleotide reductase (RNR) in *E. coli* in response to iron limitation suggests that this is one essential process that can be compromised by metal limitation \((80)\). *B. subtilis* encodes a single manganese-ribonucleotide reductase \((81)\) and a manganese-dependent phosphoglycerate mutase \((82)\), a key enzyme in glycolysis, that might each contribute to the manganese requirement for growth. Similarly, in *B. jiponicum*, pyruvate kinase has been defined as a key manganese-dependent enzyme \((83)\).

The molecular basis for manganese toxicity is also poorly understood. Manganese import into manganese-starved *B. subtilis* cells leads to a transient growth arrest \((associated with manganese hyperaccumulation)\), and growth resumes only after manganese levels return to normal \((84)\). Presumably, elevated manganese interferes with one or more critical iron-dependent enzymes. Recent results in *E. coli* suggest that ferrochelatase may be one such enzyme \((85)\), consistent with biochemical studies suggesting that Mn\(^{2+}\) can serve as a competitive inhibitor of Fe\(^{2+}\) loading into protoporphyrin IX \((86)\). In *Neisseria meningitidis*, manganese toxicity is exacerbated under low iron conditions. As a countermeasure, the MntX protein exports Mn\(^{2+}\) and increases the intracellular Fe/Mn ratio \((75)\). This and related Mn\(^{2+}\) efflux systems are important in several bacterial pathogens \((75, 87–89)\), underlining the importance of manganese homeostasis for pathogenicity.

Additional insights into the physiological effects of high manganese in *B. subtilis* have emerged from transcriptomic studies \((54)\). Shifting cells from manganese-limited to manganese-sufficient conditions led to a significant induction of the σ\(^B\) regulon apparently due to activation of the manganese-dependent RsbU phosphatase. This suggests that RsbU activity is normally limited by cofactor availability. A second notable effect was significant induction of the nitrogen limitation stress response regulated by TnrA. This was hypothesized to result from a shift of glutamine synthetase from a largely magnesium-cofactored form to a manganese-cofactored form known, from *in vitro* studies, to be less sensitive to feedback inhibition by glutamine \((54)\).

The consequences of iron starvation and iron overload have also been challenging to unravel. Starvation for iron can activate sophisticated iron-sparing responses that repress synthesis...
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of some iron-utilizing enzymes and may lead to mobilization of iron either from storage proteins or by proteolysis of dispensable metalloenzymes. The identity of the critical step(s) that ultimately fail is unclear and is likely dependent on growth conditions. In B. subtilis, and many other organisms, iron starvation leads to induction of flavodoxins that can functionally replace ferredoxins (19, 90, 91), thereby implicating the latter as being sensitive to iron deprivation. Conversely, the adverse consequences of iron overload are not well understood, although it is clear that high iron levels can enhance sensitivity to oxidative stress (72). Another possible effect of iron overload in a manganese-centric organism such as B. subtilis is interference with one or more of the critical, manganese-dependent enzymes needed for growth.

Conclusions

As this brief survey illustrates, B. subtilis provides an excellent model system for investigating the complex interactions between iron and manganese homeostasis, informed by an abundance of structural data for the cognate metalloregulators, transcriptome data revealing the responses to metal deprivation and excess, and molecular genetic studies of the corresponding gene regulatory circuitry. Studies in this and other microbial systems promise to help elucidate the complex bioinorganic chemistry of the cell, which plays a central role in host-pathogen interactions as well as elemental cycling on a global scale.

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