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Bacterial manganese sensing and homeostasis

Lauren S. Waters

Department of Chemistry, University of Wisconsin, Oshkosh, WI, 54901, USA

Abstract

Manganese (Mn) plays a complex role in the survival of pathogenic and symbiotic bacteria in eukaryotic hosts and is also important for free-living bacteria to thrive in stressful environments. This review summarizes new aspects of regulatory strategies to control intracellular Mn levels and gives an overview of several newly identified families of bacterial Mn transporters. Recent illustrative examples of advances in quantification of intracellular Mn pools and characterization of the effects of Mn perturbations are highlighted. These discoveries help define mechanisms of Mn selectivity and toxicity and could enable new strategies to combat pathogenic bacteria and promote growth of desirable bacteria.

Keywords

Manganese; Mn exporters; Mn importers; Metal homeostasis; Bacteria; MntR; *yybP-ykoY* riboswitch

Introduction

Transition metals (manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), and zinc (Zn)) are essential nutrients for bacteria, yet these metals can also be toxic in excess. The mammalian immune system takes advantage of this vulnerability and withholds critical metals, including Mn, Fe, and Zn, to starve invading bacteria in a process termed nutritional immunity [1–3]. In other situations, host defenses intoxicate bacteria with high concentrations of metals, notably Cu and Zn but also possibly Mn and Fe, during colonization of eukaryotic hosts [3–5].

Mn is required for the proper growth of many bacteria. Molecularly, Mn cofactors diverse enzymes involved in carbohydrate and nucleic acid metabolism, signaling, and oxidative stress resistance [6,7]. Mn also protects cells against oxidative stress, primarily by breaking down reactive oxygen species (ROS) (Box 1) [2,8]. However, despite these beneficial roles, excess Mn perturbs intracellular pools of other ions and causes mismetallation of important regulators and enzymes, sensitivity to ROS, and decreased virulence [5].

Corresponding author: Waters, Lauren S (watersl@uwosh.edu).

Conflict of interest statement

Nothing declared.

Therefore, to maintain an optimal Mn concentration, bacteria control intracellular Mn levels with Mn importers and exporters (Figure 1). Expression of these transporters is regulated by Mn-sensing transcription factors and riboswitches. Once inside the cell, Mn associates with various molecules, including proteins, nucleic acids, and small metabolites, in different intracellular pools [5].

This article summarizes recent findings regarding how Mn-binding regulators control gene expression, characterization of new classes of Mn exporters, the speciation and availability of intracellular Mn pools, and molecular mechanisms of Mn toxicity. Specifically, I review recent studies of (a) Mn regulators, including MntR oligomerization and the metal selectivity of the Mn-binding *yybP-ykoY* riboswitch; (b) newly identified Mn exporters; and (c) quantification and perturbation of intracellular Mn pools.

Regulators: Mn-binding transcription factors and riboswitches

Bacteria have two main mechanisms to regulate the expression of genes encoding Mn homeostasis proteins: Mn-binding transcription factors and a Mn-binding riboswitch (Figure 1).

The major transcriptional regulator controlling bacterial Mn homeostasis is the DtxR family protein, MntR [2,5]. In the absence of metal binding, the MntR apoprotein is found as a dimer in solution. Mn binding to MntR causes an allosteric change that allows it to bind DNA. Although noncognate metals bind MntR *in vitro*, they do not typically enable DNA binding or mediate MntR regulation of gene expression *in vivo* [2,5].

While protein-based sensors of Mn have been well characterized over the past 20 years, an RNA-based Mn sensor, the *yybP-ykoY* riboswitch, was only recently identified [9,10]. Found mainly in bacteria, riboswitches are RNA structures present in the 5’ untranslated regions of mRNAs. These riboregulators have two conformational states that switch upon binding a ligand to affect gene expression [11,12]. Metal-binding riboswitches have been found that respond to Mg, Ni, Co, and Mn to control expression of cognate metal transporters [13,14]. Recent work has expanded understanding of how both MntR and the *yybP-ykoY* riboswitch regulate expression of Mn homeostasis genes in different bacteria.

Mn-binding transcription factors

Long known as a repressor of Mn import genes, MntR has now been shown to directly activate transcription of Mn exporters in *Bacillus subtilis* [15]. Other recent studies explored two different aspects of MntR self-interaction that impact its regulation of gene expression. In one report, crystallization of the MntR homolog MtsR in *Streptococcus pyogenes* revealed contacts between dimers at the C-terminal FeoA domain [16]. The authors generated mutants that disrupted oligomerization without affecting dimerization. Using these mutants, they demonstrated that FeoA-dependent oligomerization can be important for proper regulation of gene expression *in vitro* and contributes to virulence *in vivo* [16].

Another form of MntR oligomerization was observed in the H_2O_2-resistant bacterium *Streptococcus oligofermentans* [17]. In this case, MntR monomers formed disulfide-linked

dimers and higher order oligomers upon *in vitro* oxidation of two key Cys residues by H_2O_2. MntR mutants lacking the Cys residues showed fewer cross-linked complexes and stronger promoter binding in the presence of H_2O_2. *In vivo*, the mutants accumulated somewhat less Mn and survived less well upon H_2O_2 challenge. The authors propose that upon oxidative stress in *S. oligofermentans*, MntR is inactivated by oxidation and cross-linking of cysteines, allowing derepression of Mn importers and increased Mn uptake, which counteracts H_2O_2 toxicity [17].

It remains to be seen how widespread these oligomerization mechanisms are. The FeoA domain is not present in all MntR homologs, and the key cysteines mediating cross-linking are not conserved outside of *Streptococci* [16,17]. It will also be interesting to see whether these self-interactions affect all MntR-regulated genes similarly or whether they could contribute to a graded response to Mn and oxidative stress.

Mn-binding riboswitch

Initial experiments revealed that the *yybP-ykoY* riboswitch bound Mn at a specific site, causing a conformational change to induce gene expression [9,10]. Recent work investigated how the *yybP-ykoY* riboswitch binds Mn by comparing three versions of the riboswitch from the *mntP, alx*, and *yoaB* (denoted *ykoY*) genes [18]. The three homologs had a wide range of metal affinities. For instance, the *mntP* riboswitch bound Mn nearly 1000-fold more tightly than the *alx* riboswitch. To gain insight into the molecular mechanism of Mn selectivity, the authors crystallized three versions of the riboswitches with different Mn-binding affinities and reanalyzed the existing structure [10]. The plasticity of the metal-binding sites across the different structures suggested that ionic radius does not significantly contribute to the Mn specificity. Rather, by comparison with the structure of MntR, Mn selectivity was deduced to result from heptacoordination of the metal, which favors high-spin Mn over other metals, and the use of a softer nitrogen ligand [18].

Another study examined the *yybP-ykoY* riboswitch in *Streptococcus pneumoniae* regulating a P-type ATPase, MgtA, which was initially suggested to efflux calcium (Ca) [19]. Although the *mgtA* riboswitch was demonstrated to have a ~50-fold tighter binding affinity for Ca than Mn via isothermal titration calorimetry (ITC), it showed a significant preference for Mn over Ca for activation of gene expression using an *in vitro* transcriptional read-through assay. Supporting the functional preference for Mn, a *mgtA-lacZ* fusion showed strong induction by 20 μM Mn *in vivo* but not with 400 μM Ca or other metals [19].

Given the broad distribution of the riboswitch across bacteria in diverse ecologies, it will be important to further refine the relationship of Mn and other metals in the gene regulation imparted by the riboswitch, as well as in the activity of the gene products the riboswitch regulates, which so far have been limited to novel Mn exporters.

New families of Mn exporters

While the major classes of bacterial Mn importers have been known for some time [2,7], new families of Mn exporters are still emerging (Figure 1). The first discovered Mn exporter, the cation diffuser family (CDF) protein MntE, was identified a decade ago in

S. pneumoniae [20]. Intriguingly, despite the observation that many pathogenic bacteria require Mn during infection and the fact that MntE reduces intracellular Mn levels, loss of MntE was shown to decrease virulence [20]. Subsequent studies confirmed this in other *Streptococci* [21,22], and recent work has shown that MntE is important for virulence in another genus, *Staphylococcus aureus* [23]. In addition, two other CDF family Mn exporters, MneP and MneS, have also been identified in *B. subtilis* by virtue of their upregulation by Mn via MntR [15].

Shortly after MntE, a second class of Mn exporter was identified, MntP [24–26]. Since the *mntP* gene was preceded by the *yybP-ykoY* riboswitch, which at the time had no known ligand, this discovery prompted the studies that demonstrated that the riboswitch bound and responded to Mn [9,10]. Like MntE, the loss of MntP reduces virulence [25,26]. More recent work has identified key amino acids in MntP that likely coordinate the Mn ion as it transits through the membrane [27].

The association of the Mn-responsive riboswitch with other membrane proteins next led to the identification of two additional classes of Mn exporter: a P_{II}-type ATPase, called YoaB in *Lactococcus lactis* and MgtA in *S. pneumoniae*, and the UPF0016 family MneA in *Vibrio* species. Expression of these transporters protected against Mn toxicity and decreased intracellular Mn levels [10,19,27,28]. Mutational studies have elucidated key residues of MneA that mediate Mn efflux [27]. Both types of transporters appear to be specific for Mn export, as the YoaB/MgtA P_{II}-type ATPase was induced specifically by Mn *in vivo*, and deletion of *mneA* in *Vibrio* caused sensitivity to Mn but not other ions [10,19,28]. However, a role in Ca efflux is also possible. Both exporters have eukaryotic homologs implicated in Ca homeostasis, and MgtA was shown to affect intracellular Ca levels [19,27]. Although Fe, and increasingly Zn, is known to affect Mn levels, there may also be a hitherto unappreciated interplay between Mn and Ca homeostasis in bacteria. As for the other Mn exporters, MgtA has been implicated in pathogenesis [29], and it will be important to determine whether MneA also contributes to virulence.

Interestingly, another protein upregulated by Mn via the *yybP-ykoY* riboswitch may be an Mn importer rather than an exporter. The TerC family protein Alx could not rescue the Mn sensitivity of cells lacking MntP in *Escherichia coli*. Moreover, expression of Alx led to increased intracellular Mn levels upon Mn stress [27]. Although it seems counterintuitive for cells to upregu-late Mn import when Mn levels are already high, the *alx* gene is also induced by high pH, which decreases Mn availability [30,31]. Since TerC is the most frequently associated protein family with the *yybP-ykoY* riboswitch, it will be interesting to establish the mechanism of Alx in Mn and pH homeostasis and determine if this role is typical of all TerC homologs [Note added in proof: A newly published study has demonstrated that two TerC homologs in *B. subtilis* not associated with pH regulation likely serve as Mn exporters [32].]

It is also noteworthy that while MntP is controlled by Mn both transcriptionally and post-transcriptionally through MntR and the *yybP-ykoY* riboswitch, this dual regulation is not found for the other Mn exporters (Figure 1). MneA, YoaB/MgtA, and TerC are not regulated by a MntR homolog, and the CDF family exporters are not associated with the *yybP-ykoY*

riboswitch [9]. It is not yet clear why some Mn exporters are controlled by Mn-binding transcription factors and others by Mn-sensing riboswitches, but may relate to different physiological roles of the exporters, or ecological niches of the bacteria in which they are found [27].

Quantification of intracellular Mn pools and metal selectivity

Regulated expression of importers and exporters controls the total intracellular levels of metals, called the quota. Importantly, however, metals are partitioned into different pools once inside of the cell (Figure 1, Box 2). Methods to robustly measure these different Mn populations have been challenging to develop. Two main approaches used to quantify available metal pools have been electron paramagnetic resonance (EPR) spectroscopy and thermodynamic models to determine the sensitivities of regulatory proteins for different metals [5,33–35]. Recent work has continued to refine these approaches and provided insight into Mn speciation, availability, and toxicity within bacterial cells [6,36,37].

Speciation of Mn inside cells

The Daly and Hoffman groups have defined a specific EPR signal that represents Mn bound to low molecular weight (LMW) compounds [36]. Such LMW compounds have been shown to help protect cells against oxidative stress and ionizing radiation (IR) (Box 1) [2,8,35,38]. While ROS- and IR-resistant bacteria accumulate higher quotas of Mn than ROS- and IR-sensitive bacteria [39], the amount of Mn present in the LMW ROS-scavenging complexes relative to the total intracellular Mn has not been clear. Using absorption-display EPR in live cells, the authors were able to distinguish two populations of intracellular Mn: (1) $HeMn^{2+}$, high-symmetry LMW Mn complexes associated with resistance to IR and (2) $L\text{-}Mn^{2+}$, low-symmetry Mn complexes. The ratio of $H\text{-}Mn^{2+}$ to $L\text{-}Mn^{2+}$ complexes in various organisms correlated well with the DNA repair efficiency of irradiated cells. The authors propose that this ratio in different cell types—including human cancers—can thus be used as a direct metric of radiation and ROS resistance [36].

Available metal pools predict metal selectivity *in vivo*

A recent comprehensive study inferred the available metal pools in *Salmonella* cells from measurements of binding affinities and concentrations of seven metal-sensing transcription factors and the metals Mn, Fe, Co, Ni, Zn, and Cu [37]. Using a thermodynamic model, they derived the available concentration of each metal and calculated the free energy of metallation ($\Delta G°$) for the transcription factors with their cognate metal (Box 2). Importantly, the authors then showed that actual metal specificity of an example protein was predicted by the available amount of metals rather than the *in vitro* metal-binding affinities, as is often used. This and previous studies suggest that metal concentrations which exceed the buffering capacity of the cell could cause mismetallation [5,34,37]. Thus, this type of analysis could be used to analyze perturbations in metal pools on mismetallation. Although it would require significant effort, similar systematic quantification of available metal pools in different bacteria and under different growth conditions would be illuminating.

Toxicity due to perturbation of metal pools

The excess or deficiency of metals has long been known to inhibit bacterial growth. However, the specific cellular targets that are mismetallated or under-metallated and how concentrations of other metals are perturbed have been less clear [5]. Recently, a new target of Mn toxicity was identified in *S. pneumoniae* [6]. High Mn concentration led to hyperactivation of the protein phosphatase PhpP, which inhibited cell division. Interestingly, while high Mn concentrations have been associated with decreased Fe levels in *E. coli* and *S. aureus*, in this study, excess Mn led to increased Fe and Zn levels [6,23,40]. *In vitro*, Zn strongly inhibited activity of PhpP, suggesting that controlling the Mn:Zn ratio is important for proper cell functioning. Further studies are needed to define other cellular targets of Mn intoxication and deficiency across bacteria.

Perspective

In summary, significant progress has been made in understanding how Mn transporters and Mn-binding regulatory factors work, including recent work on Mn-dependent gene regulation, identifying new Mn homeostasis proteins, and characterizing Mn pools in cells. But by analogy with other metal homeostasis systems, dedicated factors that sequester excess Mn or that mobilize intracellular stores during Mn deficiency may also exist. Possible candidates include the MntR-regulated Dps protein and other genes associated with the *yybP-ykoY* riboswitch [7,9,41]. Additionally, while the importance of Mn for virulence of pathogenic bacteria is becoming even more apparent, similar work establishing the role of Mn during symbiotic interactions with eukaryotic hosts or within bacterial communities is mostly lacking. An exception is a recent study that provided mechanistic insight into Mn usage during colonization of *Rhizobium leguminosarum* with various plants [42]. Understanding the broader role of Mn in beneficial interactions of bacteria will also be an exciting area to follow in upcoming years.

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Box 1.

Mn and oxidative stress

Manganese (Mn) protects cells from oxidative stress via several distinct mechanisms [2,7,8]. Mn serves as a cofactor for enzymes that break down reactive oxygen species (ROS), mainly Mn superoxide dismutase (SOD) and Mn-cofactored catalases and peroxidases. *In vitro*, Mn can also nonenzymatically convert superoxide ($O_2^{\bullet -}$) to H_2O_2 and H_2O_2 to water when complexed with small molecule metabolites such as phosphate, bicarbonate, or certain amino acids [43–45]. Additionally, Mn has been shown to substitute for Fe in some mononuclear Fe enzymes in *Escherichia coli* under oxidative stress, protecting the enzymes from Fenton reaction–mediated oxidative damage while still retaining catalytic activity in the Mn-bound form [46,47]. The relative import of these different ROS protection mechanisms in different bacteria is an active area of research.

Alterations in total intracellular Mn levels can alter ROS sensitivity. For example, loss of Mn importers typically increases sensitivity of pathogenic and symbiotic bacteria to oxidative stress (as most recently shown in Refs. [42,48,49]). Interestingly, loss of Mn exporters, which leads to increased Mn levels, also affects ROS resistance, but has been shown to increase or decrease sensitivity to oxidative stress depending on the bacteria and the specific type of stress [6,20–23,25,27,28].

Box 2.

Intracellular metal pools and metal selectivity

The idea that different populations of metals exist within cells has been appreciated for quite some time; however, identifying and quantifying these pools has proved challenging [5,33,34,50]. The terms “available” or “buffered free” metal describe the labile pool kinetically accessible to proteins, while the total amount of metal in cells is called the “metal quota.” Metal quotas are typically measured with inductively coupled plasma mass spectrometry (ICP-MS); however, quantification of the available metal pools has been difficult to experimentally interrogate [50].

Besides direct detection techniques using spectroscopy (challenging for some metals, including Mn), thermodynamic models have been employed to determine available metal concentrations indirectly. This approach uses the affinity of metalloregulatory factors to infer the cytoplasmic set point for each metal [34]. The binding affinity (K_A) of a regulator for its cognate metal can then be converted to the $\Delta G°$ of metallation. Since metalloregulators are the first responders in the battle to avoid excess metals, their free energy of metallation should represent the lower bound for a protein to obtain a given metal from cytoplasmic ligands [33,37]. Thus, if a protein has a less favorable free energy of metallation with that metal than this lower bound, it is not likely to obtain that metal from the available pool.

Another challenging topic to investigate continues to be how proteins and other molecules correctly select their cognate metal ion from among all other metals [50]. Differently from other biomolecules where intermolecular forces between complementary binding surfaces dictate strength of binding, divalent metals have been observed to show a stereotypical order of stability for complex formation with organic ligands, called the Irving–Williams series. Mg and Ca tend to bind most weakly, followed by Mn < Fe < Co < Ni < Cu > Zn [34,50–52]. Thus, among transition metals, Mn typically has the lowest intrinsic affinity for biomolecules. (A notable exception is unusually high stability complexes of Mn with nucleic acids [53].) This means that even for a bona fide Mn-binding protein, Mn will usually bind less tightly to the protein than Zn. (It should be noted, however, that kinetic trapping of a metal to a protein, for example, by protein folding, can increase the binding of a metal to low-affinity sites [37,52].) Thus, mismetallation of proteins with Zn and Cu is a constant problem for cells. Even Mn can become inappropriately associated with ligands, especially Fe proteins, if its levels increase and exceed the buffering capacity of cells [2,5,37,40]. Defining which proteins are affected by perturbations in metal pools to understand mechanisms of metal toxicity is an ongoing effort, and an important one. Emerging evidence suggests this “metal specificity problem” is an inherent aspect of metalloproteins and is exploited by eukaryotic hosts by subjecting bacteria to metal intoxication or starvation to kill invading pathogens [4,37].

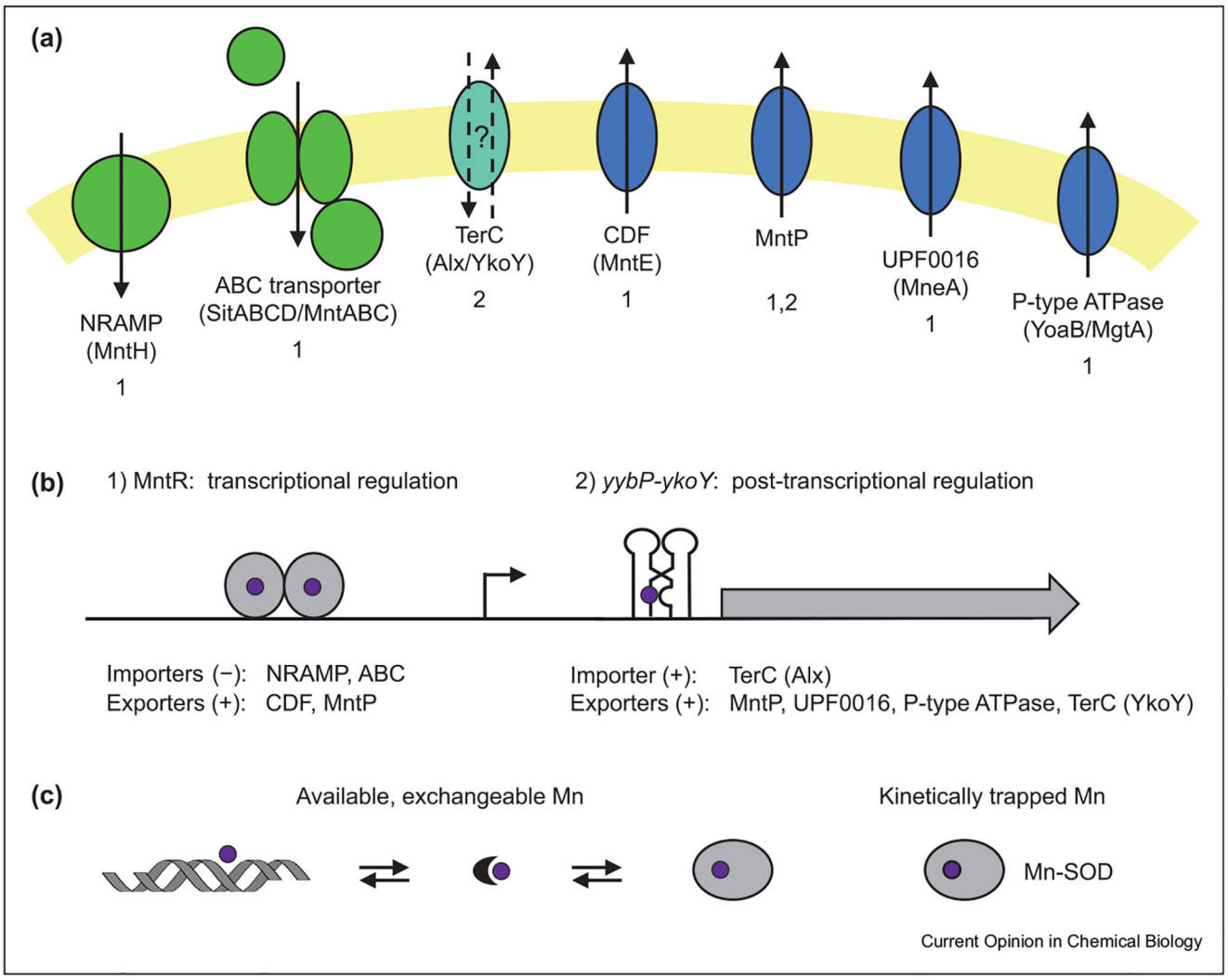

Figure 1. Manganese homeostasis proteins across bacteria and their regulation by Mn.

The complement of common Mn importer and exporter families in bacteria and their typical regulation by a transcription factor or a riboswitch. **(a)** Two major classes of Mn importers (green) are the NRAMP family, exemplified by MntH, and ABC transporters, the Mn-specific versions of which have various names in different species, commonly SitABCD in gram-negative bacteria and MntABC in gram-positive bacteria. Four major classes of exporters (blue) used to efflux Mn are the CDF, MntP, UPF0016, and P-type ATPase families, with specific protein examples as indicated. The TerC family proteins (blue-green) may function as a Mn importer or exporter depending on the organism [27,32]. Other rare Mn transporters are not shown, including the importers MntX (ArsP_2 type), MntA (P-type ATPase), and the exporter CtpC (P-type ATPase) [54–56]. The typical mechanism of regulation is indicated by a 1 or 2 below the protein names. **(b)** Two main mechanisms are employed to regulate gene expression of Mn transporters: (1) transcriptional regulation via MntR (or sometimes Mur [57], not shown) or (2) post-transcriptional regulation via the *yybP-ykoY* riboswitch. A + sign indicates upregulation, and a − sign indicates downregulation. Other metal homeostasis proteins impacted by these regulators are not

shown, but often include Fe importers upregulated in various bacteria by MntR and several families of proteins of unknown function induced by the riboswitch. **(c)** Once inside cells, Mn (purple) is partitioned into different intracellular pools, including Mn bound to proteins, DNA and RNA, small molecule chelates (crescent), and possibly some fully hydrated ions. The total Mn quota consists of Mn ions that are weakly bound and exchangeable, as well as kinetically trapped Mn (e.g., buried in proteins such as Mn-SOD or MncA [2,52]).