The *Escherichia coli* MntR Miniregulon Includes Genes Encoding a Small Protein and an Efflux Pump Required for Manganese Homeostasis\(^\dag\)†

Lauren S. Waters, Melissa Sandoval, and Gisela Storz*

*Cell Biology and Metabolism Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, Maryland*

Received 26 July 2011/Accepted 28 August 2011

Manganese is a critical micronutrient for cells, serving as an enzyme cofactor and protecting against oxidative stress. Yet, manganese is toxic in excess and little is known about its distribution in cells. Bacteria control intracellular manganese levels by the transcription regulator MntR. When this work began, the only *Escherichia coli* K-12 gene known to respond to manganese via MntR repression was *mntH*, which encodes a manganese importer. We show that *mntS* (formerly the small RNA gene *ryb4*) is repressed by manganese through MntR and encodes an unannotated 42-amino-acid protein. Overproduction of MntS causes manganese sensitivity, while a lack of MntS perturbs proper manganese-dependent repression of *mntH*. We also provide evidence that *mntP* (formerly *yebN*), which encodes a putative efflux pump, is positively regulated by MntR. Deletion of *mntP* leads to profound manganese sensitivity and to elevated intracellular manganese levels. This work thus defines two new proteins involved in manganese homeostasis and suggests mechanisms for their action.

Manganese is a critical micronutrient for organisms from bacteria to humans. Due to its particular redox properties, manganese is an excellent catalyst for a wide array of chemical reactions (23, 27). Manganese also binds to a variety of small molecules, such as nucleotides and amino acids (6, 21), and serves a structural role in the stability of various macromolecular structures (23, 27).

Manganese is a cofactor for enzymes with diverse functions. Two well-studied catalytic roles are superoxide dismutation by manganese superoxide dismutase (SOD) and oxygen production by the manganese complex of photosystem II (23). Additionally, manganese is used by regulatory proteins, such as cyclic-di-GMP phosphodiesterases, and several enzymes involved in central carbon metabolism (23, 27, 37). However, the contribution of manganese to protein activity is still being revealed. Since enzymes often show activity with a range of metals in *vitro*, the physiologically relevant metal in *vivo* is unclear for many proteins. For example, the NrdEF isoform of ribonucleotide reductase, long known to be active with a different metallocofactor in *vitro*, was recently demonstrated to use manganese in *vivo* and in *vitro* (4, 31). Additionally, emerging evidence suggests alternative metals can be used by enzymes under different conditions. The ribulose-5-phosphate 3’ epimerase, previously known to use iron, was recently shown to also employ manganese to retain activity under conditions of oxidative stress (42). More enzymes likely will be found that use manganese in *vivo* either constitutively or selectively under certain conditions.

It has been established that manganese is an important element in the defense against oxidative stress. Bacterial mutants deficient in manganese transport show increased sensitivity to reactive oxygen species (1, 20, 28, 39) and a reduced ability to survive during pathogenic and symbiotic interactions (11, 37). High intracellular concentrations of manganese are correlated with increased resistance to ionizing radiation (8) and desiccation (14), apparently protecting proteins from oxidation (7). Manganese detoxifies reactive oxygen species, both by stimulating antioxidant enzymes, including superoxide dismutases, catalases, and peroxidases, and by nonenzymatic mechanisms (6, 21). In addition, manganese was recently proposed to substitute for and displace iron from the active sites of mononuclear iron proteins, thus preventing oxidative damage to the proteins (1, 42).

Despite serving essential cellular roles in enzymatic catalysis and protection against oxidative stress, manganese is harmful in excess. The molecular mechanisms of manganese toxicity in bacteria are not clear; however, they most likely involve perturbing iron metabolism (16). Additionally, elevated manganese levels could affect the activities of enzymes dependent on other metals (27).

Bacteria are mostly protected from the consequences of under- or overaccumulation of manganese by homeostasis systems, which are typically composed of a manganese-dependent transcription regulator, manganese transporters, and a recently identified manganese efflux pump (41, 44). The MntR transcription factor serves as the primary sensor and transducer of manganese abundance. Upon binding to manganese, MntR binds promoter DNA to repress or activate transcription of its target genes (15, 30). In enterobacteria, the only genes known to be regulated by MntR when this work began were *mntH* and

\[^{\dag}\text{†}\text{†}\] Supplemental material for this article may be found at http://jb.asm.org/.

\[^{\dag}\text{†}\text{‡}\] Published ahead of print on 9 September 2011.
sitABCD, encoding manganese transporters, of which laboratory strains of *Escherichia coli* only possess *mntH* (22, 26, 38). In some Gram-positive bacteria, the MntR analog seems to regulate a large set of genes and mediate a more global response (36). In other Gram-positive bacteria, such as *Bacillus subtilis*, MntR appears to directly regulate only manganese transporters (*mntH* and *mntABCD*). However, manganese can associate with the PerR transcription factor to regulate other genes (16).

We performed whole-genome expression analysis of wild-type and ΔmntR strains of *E. coli* and uncovered two new genes of previously unknown activity that participate in manganese homeostasis. Expression of *mntS* (formerly *rybA*) is repressed by manganese via MntR. Unexpectedly, the *mntS* gene was found to encode a novel small protein of 42 amino acids. We also discovered that the *mntP* gene (formerly *yebN*), encoding a putative efflux pump, is upregulated by manganese through MntR. The phenotypes associated with deletions of *mntS* and *mntP* as well as overproduction of MntS support roles in intracellular and extracellular manganese trafficking. These results expand our knowledge of manganese homeostasis and the MntR regulon in Gram-negative bacteria.

### MATERIALS AND METHODS

**Strains and plasmids.** Strains used in this study are listed in Table S1 of the supplemental material, and the sequences of oligonucleotides used are given in Table S2 of the supplemental material. Chromosomal deletion strains were generated using pKD4 or pKD13 (10) via mini-

**Metal sensitivity assay.** For the experiment shown in Fig. 5A, strains were grown overnight in LB with ampicillin and arabinose for the plasmid-bearing strains) containing the indicated concentrations of metals. For the experiment shown in Fig. 5B, strains were grown overnight in LB medium, diluted 1:2000 in LB, grown to an OD600 of ~0.5, and spotted onto plates as described above.

**ICP-MS.** For inductively coupled plasma-mass spectrometry (ICP-MS), wild-type and ΔmntR strains were grown overnight in LB medium, diluted 1:4000 in LB, grown to an OD600 of ~0.3, and spotted onto an OD600 of ~0.15 in 1.5 L of LB containing 500 μM MnCl2. Cells were grown for 2 to 2.5 h to an OD600 of ~0.23. The ΔmntR strain began exhibiting slower growth ~2 h after dilution into high-manganese medium. Cells were harvested and prepared for analysis as described previously (1). The manganese concentrations were determined with a SCIEX ELAN DRCe apparatus (Perkin-Elmer) at the University of Illinois Microanalysis Laboratory and normalized to total protein in the lysates as described previously (1). The values given are averages of three independent replicates.

**RESULTS**

Whole-genome expression analysis revealed new genes regulated by MntR. In our search to characterize orphan small RNA (sRNA) genes without a known function, we became interested in the *rybA* gene, which shows strong synteny with the gene encoding the manganese-dependent transcription factor MntR across enterobacteria (Fig. 1A). Since transcription regulators in bacteria are often encoded divergently from the genes they regulate, we wondered whether *rybA* was a member of the MntR regulon. To address this, we performed whole-genome expression analysis using microarrays. Wild-type and ΔmntR cells were grown in minimal medium to mid-exponential phase and exposed to 10 μM MnCl2 for 1 h. Expression corresponding to the region of the *rybA* gene showed strong derepression in the ΔmntR strain (~100-fold), indicating *rybA* is indeed negatively regulated by MntR. Since *rybA* was encoded next to and regulated by *mntR*, we renamed the gene *mntS*. The only other gene strongly derepressed was *mntH*.
FIG. 1. The mntS locus, sequence alignment, and transcript profile. (A) Schematic of the mntS genomic locus. (B) Alignment of the mntS gene, showing the consensus MntR binding motif (purple), predicted −10 and −35 promoter regions, mapped transcription start site (1) at position 852270 in the MG1655 genome, consensus Shine-Dalgarno (SD) sequence, MntS open reading frame (ORF; yellow), and the first 3′ end of the mntS transcript at position 852065. Species included Escherichia coli, Shigella boydii, Salmonella enterica, Citrobacter koseri, Klebsiella pneumoniae, Enterobacter sakazakii, and Cronobacter turicensis. (C) Northern blot analysis of the mntS transcript from wild-type (MG1655) and ΔmntS (GSO459) cells grown to mid-exponential phase in M9-glucose medium.
TABLE 1. Genes that changed >5-fold in both arrays

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold changea</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>mntS</td>
<td>0.01</td>
<td>Microarray 1</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>Microarray 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Putative manganese</td>
</tr>
<tr>
<td></td>
<td></td>
<td>chaperone</td>
</tr>
<tr>
<td>mntH</td>
<td>0.13</td>
<td>Putative manganese</td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>transporter</td>
</tr>
<tr>
<td>mntP (yebN)</td>
<td>5.9</td>
<td>Putative manganese</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>efflux pump</td>
</tr>
<tr>
<td>mntP (yebN) Ig region</td>
<td>5.7</td>
<td>Putative riboswitch</td>
</tr>
<tr>
<td></td>
<td>10.2</td>
<td>upstream of efflux pump</td>
</tr>
</tbody>
</table>

a Microarray 1 represents gene expression data from cells incubated with 10 μM MnCl₂ for 60 min, while microarray 2 represents gene expression data from cells incubated with 10 μM MnCl₂ for 10 min. The fold change represents the signal from the wild-type culture (MG1655) relative to the signal from the ΔmntR culture (GSO458).

(~8-fold), encoding the MntH manganese transporter and known to be MntR regulated (26, 38). In contrast, many genes showed somewhat higher expression in the wild-type strain relative to the ΔmntR strain (GEO accession number GSE25318), likely due to indirect effects arising from an extended imbalance of manganese homeostasis.

To identify genes positively regulated by MntR, we performed the microarray analysis with RNA extracted from wild-type and ΔmntR cells exposed to 10 μM MnCl₂ for 10 min (GEO accession number GSE25318). Only one gene, yebN, was strongly upregulated in the wild-type strain relative to the ΔmntR strain in the arrays from both time points (Table 1).

We also observed some derepression of many iron-responsive genes in the ΔmntR strain compared to the wild-type strain after 10 min in 10 μM MnCl₂. Of the top 20 genes (>5-fold repressed) present at higher levels in the ΔmntR strain, 15 (75%) were iron regulated (see Table S4 in the supplemental material) and of the top 60 genes (>3-fold repressed), 24 (40%) were iron regulated. These genes corresponded to almost all of the known iron transporters and siderophore biosynthesis genes. This suggests that cells overloaded for manganese, as in the case of ΔmntR, perceive a condition of limited iron, consistent with the proposed displacement of iron by manganese (1).

The mntS gene has multiple transcripts. The mntS gene was identified in a computational screen for sRNAs (48) and was confirmed by Northern blotting with a riboprobe spanning the intergenic region between mntR and yilL. We wanted to map the boundaries of the mntS gene, since the precise location and sequence of the gene were unknown. Primer extension analysis revealed that the mntS transcript has a single 5’-end, while 3’-random amplification of cDNA ends (RACE) analysis showed that the transcript has multiple 3’ ends (see Table S3 in the supplemental material), with the first end corresponding to a 205-nucleotide (nt) transcript (Fig. 1B). We also observed seven predominant bands from our Northern analysis (Fig. 1C). The transcription start site mapped just downstream of MntR consensus sites, consistent with MntR repression.

Surprisingly, the mapped location of the mntS transcript overlapped with the predicted yilL gene present on the opposite strand, suggestive of cis-encoded antisense regulation. However, upon closer investigation, the yilL gene appeared to be spurious. The YilL protein is poorly conserved, lacks an obvious Shine-Dalgarno sequence, and does not show significant homology to any other proteins. We tried unsuccessfully to detect the yilL transcript by Northern analysis with three different DNA probes and a riboprobe, as well as by 3’-RACE (data not shown). We also were unable to detect an epitranscript version of the YilL protein (see below). These data clarify the gene structure of this part of the chromosome; there is a complex set of transcripts for mntS, but no apparent expression of yilL.

mntS is downregulated by manganese and MntR. To confirm repression by manganese via MntR, we examined mntS and mntH expression using Northern analysis (data not shown) and primer extension assays (Fig. 2). The mntS transcript was present at high levels during exponential-phase growth in M9 minimal medium but was robustly downregulated by addition of manganese. This repression was completely lost in a ΔmntR strain, validating the microarray results. In LB rich medium, mntS was present at lower levels but could be induced modestly by addition of the divalent cation chelator EDTA in wild-type cells, but not in the ΔmntR strain.

As previously demonstrated (22, 26, 28, 38), we observed that mntH is repressed by manganese and induced by EDTA (Fig. 2). This analysis experimentally confirmed that the mntH mRNA has a single 5’-end at the predicted location (see Fig. S1 in the supplemental material) (26, 28). In the ΔmntR strain, both the repression of mntH by manganese and the induction by EDTA were reduced. However, residual regulation by man-
ganese and EDTA in the ΔmntR strain was still observed. This remaining regulation likely is due to mntH repression by Mnt-loaded Fur protein, given that manganese repression is completely lost in a ΔmntR Δfur double mutant background (22, 26).

mntS expression is not directly affected by iron. The levels of mntS are only modestly repressed by iron (Fig. 3). The slight iron repression of mntS is completely lost in the ΔmntR strain. This suggests that iron can repress mntS through Fe-MntR, similar to mntH repression by Mn-Fur. Consistent with a lack of direct regulation by Fur, we did not observe a consensus Fur binding site in the mntS promoter (Fig. 1B). Thus, distinct from mntH, mntS is uniquely controlled by MntR, suggesting the mntS gene is needed specifically when manganese levels are low. Although not downregulated by iron, the mntS transcript is repressed by 2,2′-dipyridyl, which is commonly used to chelate iron but also has affinity for copper and zinc (Fig. 3). The decrease in mntS levels is not due to increased repression by MntR, since the 2,2′-dipyridyl repression of mntS is still observed in the ΔmntR strain.

As a control, we also monitored mntH and, as previously reported, observed that mntH was repressed by the addition of iron and induced by treatment with 2,2′-dipyridyl, consistent with repression by Fur (Fig. 3). The repression of mntH by iron was unaffected by the deletion of mntR, as expected. However, mntH induction by 2,2′-dipyridyl treatment was increased in the ΔmntR strain, due to an effective lack of both Fur and MntR activities.

In summary, we found that mntS is repressed by manganese in an MntR-dependent manner but, unlike mntH, is not directly affected by iron. However, mntS expression is subject to additional, as yet undescribed levels of regulation.

The mntS gene encodes a small protein. Upon further inspection of the mapped mntS transcript, we unexpectedly observed a conserved small open reading frame of 42 amino acids preceded by a consensus Shine-Dalgarno sequence (Fig. 1B and 4A). Although the protein has many well-conserved hydrophobic amino acids, they do not appear to form a transmembrane region, a feature common to many other small proteins (18). We verified expression of a sequential peptide affinity (SPA)-tagged version of the MntS protein (Fig. 4B) and observed two bands, suggesting the small protein may be modified or exist in two distinct conformations. Under all conditions tested, we did not detect an SPA-tagged derivative of the oppositely encoded YliL protein.

MntS overproduction causes sensitivity at elevated manganese levels. Since cells repress MntS expression when manganese is present, we wondered whether high levels of MntS are detrimental to cells exposed to high concentrations of manganese or other cations. Indeed, elevated amounts of MntS caused significant sensitivity in the presence of manganese (Fig. 5A). This phenotype was only observed with manganese, as overproduction of MntS did not cause sensitivity to an equivalent dose of zinc or to a 100-fold-higher dose of magnesium (Fig. 5A), nor did it do so with iron [Fe(II) and Fe(III)], nickel, copper, cobalt, or cadmium (data not shown).

The observation that overproduction of MntS causes manganese sensitivity suggests that MntS functions to specifically make manganese more available in cells.

Lack of MntS perturbs proper repression of mntH. Although we did not observe a growth phenotype for the ΔmntS strain (Fig. 5A), we wondered whether loss of MntS would affect intracellular manganese pools and alter repression of mntH. To examine this, we monitored regulation of mntH transcript levels in wild-type and ΔmntS strains grown with increasing concentrations of supplemental manganese. Consistent with previous studies (22, 26), mntH levels were repressed...
in wild-type cultures grown in minimal medium with concentrations of supplemental manganese of 1 \( \mu \text{M} \) and above (Fig. 6). In contrast, in the \( \Delta \text{mntS} \) strain, \( \text{mntH} \) levels failed to show full repression by manganese, even at concentrations of supplemental manganese up to 1 mM. This indicates that MntS promotes the ability of manganese to repress \( \text{mntH} \) transcription.

The levels of the \( \text{mntS} \) transcript also decreased with increasing manganese, showing significant repression by 1 \( \mu \text{M} \) MnCl\(_2\) (Fig. 6). However, we were able to detect the MntS-SPA fusion protein, albeit at very low levels, even at 1 mM manganese. This suggests that at high extracellular manganese concentrations, \( \text{mntS} \) is repressed but still produces sufficient MntS protein to provide an activity required for the cell to efficiently repress \( \text{mntH} \) transcription. This result also is consistent with the idea that MntS affects cellular manganese availability.

The \( \text{mntP} \) gene is upregulated by manganese and MntR. We next turned our attention to \( \text{yebN} \), the gene from the microarray that appeared to be positively regulated by MntR. The \( \text{yebN} \) gene had previously been suggested to have an MntR binding site (22, 26), as well as a Fur motif (43) and an uncharacterized riboswitch regulatory element (2, 25) (Fig. 7).
The protein also has distant but significant similarity to several LysE family efflux pumps (expect values, \(10^{-11}\) to \(10^{-14}\) using HHpred), including the RcnA nickel/cobalt efflux pump (40). In light of the data presented below, we renamed this gene \(mntP\).

We mapped the transcription start site of \(mntP\) by primer extension analysis to within 5 nt (Fig. 7B and 8A). The \(mntP\) is located 150 nt and 190 nt downstream of the two conserved MntR consensus sites, ~80 nt downstream of the conserved Fur motif, immediately upstream of the riboswitch homology region, and ~225 nt upstream of the MntP start codon. During the course of this work, a global chromatin immunoprecipitation-microarray chip (ChIP-chip) analysis showed that MntR indeed binds at the predicted sites (49). While the MntR consensus sites at the \(mntS\) and \(mntH\) genes...
to manganese (Fig. 5B), consistent with an inability to pump excess metal out of cells. The $\Delta mntP$ sensitivity was significantly stronger than the sensitivity observed for $\Delta mntR$. The $\Delta mntP$ and $\Delta mntR$ phenotypes were specific to manganese, as no growth inhibition was observed with an equivalent dose of zinc or with a 100-fold-higher dose of magnesium (Fig. 5B) or iron [Fe(II) and Fe(III)], nickel, or copper (data not shown). In contrast to $\Delta mntP$ and $\Delta mntR$, the $\Delta mntH$ and $\Delta mntS$ strains showed no differences in wild-type cells in growth on plates containing elevated manganese. This was not unexpected, since $mntH$ and $mntS$ are not expressed under conditions of high manganese. The specificity and severity of the $\Delta mntP$ growth defect in the presence of high manganese suggest that $\Delta mntP$ cells accumulate excess manganese due to a lack of efflux activity. Consistent with this suggestion, heterologous MntP expression rescued the $\Delta mntR$ sensitivity and even increased wild-type resistance to manganese (data not shown).

**Deletion of mntP leads to increased intracellular manganese levels.** We next directly assayed the intracellular manganese levels in $\Delta mntP$ and wild-type cells by ICP-MS. $\Delta mntP$ cells grown in LB containing 500 $\mu$M MnCl$_2$ accumulated 2.3-fold more manganese than wild-type cells (47.9 ± 6.0 $\mu$M compared to 21.1 ± 1.4 $\mu$M, similar to the 15 $\mu$M concentration measured for wild-type cells previously [1]). This is consistent with the ~3- to 4-fold increase in intracellular manganese levels observed in strains lacking MntE, an unrelated manganese efflux pump in Gram-positive bacteria (41, 44). Taken together, the observations that $mntP$ is upregulated by manganese through MntR while its deletion causes dramatic sensitivity to manganese and increased intracellular manganese levels indicate that MntP functions as an efflux pump.

**DISCUSSION**

In this study, we characterized the MntR regulon in *E. coli* and identified two new proteins required for manganese homeostasis. We discovered the 42-amino-acid MntS protein, whose expression is repressed by manganese in an MntR-dependent manner and causes manganese toxicity when overproduced. Additionally, loss of MntS affects normal manganese-dependent regulation of the $mntH$ gene, which encodes a manganese transporter. *E. coli* contains >65 small proteins of <50 amino acids that are thought to play important roles in cell physiology yet which are mostly uncharacterized to date (18). This study provides insights into how small proteins can be incorporated into environmental response pathways. Given that $mntS$ was originally identified as the RybA sRNA (48), other genes initially annotated as noncoding or sRNAs also may encode unrecognized small proteins.

We additionally found that expression of the $mntP$ gene is upregulated by manganese through MntR. Deletion of $mntP$ confers a striking sensitivity specifically to manganese and causes aberrantly high intracellular manganese accumulation. The MntP protein has homology to the LysE family of efflux pumps but not to the previously identified MntE manganese efflux pump, a member of the cation diffuser family (41, 44). These data strongly suggest that MntP functions as a new class of manganese efflux pump.

**The MntR regulon includes mntS and mntP.** Our microarray analysis showed that expression levels of the previously unchar-
acterized genes mntS and mntP are regulated by MntR. While this work was in progress, another study reported ChiP-chip data that identified the genomic binding sites of MntR in *E. coli*, which complements our findings of the MntR regulation of mntS and mntP (49). The direct MntR regulon appears to be small, in agreement with our data and a previous prediction (26). Interestingly, in addition to sites in the promoters of mntH, mntS, and mntP, Yamamoto et al. (49) identified an MntR site upstream of dps, which encodes a ferritin-like iron storage protein, and showed that MntR represses dps expression, primarily in stationary phase. We did not observe a significant change in dps levels in our microarray analysis, likely because we used exponentially growing cells.

In addition to genes that MntR directly binds, we noted that many iron acquisition genes are derepressed in the ΔmntR strain, in which manganese levels should be aberrantly high (see Table S4 of the supplemental material). At first glance, this is surprising, since Mn-loaded Fur can repress expression of at least some Fur-regulated genes (22, 26). In addition, it is thought that iron displaced from proteins by excess manganese can bind Fur to elicit Fur-dependent repression (16). Our observation indicates that at certain intracellular levels, manganese can repress iron acquisition gene expression but that at abnormally high concentrations, manganese can derepress these same genes. The cross-regulation observed between the two metals emphasizes the complex relationship between manganese and iron in vivo.

mntS and mntP are subject to additional levels of regulation.

The levels of mntS are controlled by MntR, but our evidence also supports further regulation. In LB, mntS levels were slightly increased in the ΔmntR strain, but not to the same extent as seen in M9 medium (Fig. 2, compare lanes 5 and 7 with lane 1). Also, mntS levels are not significantly increased in LB in the ΔmntH strain relative to wild type (data not shown), despite the fact that ΔmntH cells have low levels of intracellular manganese (1), which would disable repression by Mn-MntR. Fur is not likely to regulate mntS directly, since there was no iron regulation independent of MntR (Fig. 3, lane 4). Moreover, expression of Fur-regulated genes usually increases in response to 2,2′-dipyridyl, yet mntS levels decreased, even in a ΔmntR strain (Fig. 3, lanes 5 to 8). Taken together, these results indicate mntS is controlled by a factor or factors in addition to MntR and distinct from Fur. This regulation could be at the level of RNA stability or processing.

Although the potential for cis-encoded antisense regulation of mntS by the ylfL transcript exists (Fig. 1A), we were unable to detect any ylfL transcript or protein. One study employing microarrays suggested that the ylfL transcript is upregulated by blue light and cold shock; however, its expression was never confirmed (45). Since we could not detect ylfL expression under similar conditions, we conclude that it is likely to be a pseudogene in the strain we examined. Therefore, even if an RNA opposite to mntS were transcribed under certain conditions, under normal growth in LB or M9 media its levels would be extremely low and not likely to contribute to mntS regulation. Given the unusual ladder of 3′ ends of the RNA, other types of regulation occurring at the 3′ region of the mntS transcript remain an intriguing possibility.

We demonstrated MntR-dependent upregulation of mntP upon exposure to manganese (Fig. 8), indicating that in addition to repressing gene expression, MntR can positively regulate transcription. However, the mntP transcript and protein both still showed a small amount of manganese-dependent activation in the ΔmntR strain. Since there is a predicted Fur binding site in the mntP promoter, one possibility is that, akin to the partial repression of mntH by Mn-loaded Fur, Mn-Fur partially activates mntP transcription. This seems unlikely, since direct activation of transcription by Fur in *E. coli* has not been shown conclusively but rather occurs indirectly through the RybB sRNA or H-NS protein (29, 32, 33). Another possibility is riboswitch-mediated posttranscriptional or translational regulation of the mntP gene.

The mntP gene is preceded by a predicted riboswitch element (2) that is homologous to the riboswitch present upstream of the alx gene. In the case of alx, transcriptional pausing caused by elevated pH is thought to allow the formation of alternative hairpins in the nascent mRNA, causing inhibition of alx translation (34). Intriguingly, the authors also found that addition of manganese, which is known to alter the activity of RNA polymerase, reduced pausing and affected the conformation of the riboswitch in vitro. It seems plausible that the mntP riboswitch may also respond to manganese or a related small molecule to modulate levels of the putative manganese eﬄux pump. Ongoing studies are directed toward testing this model, which, if correct, would define a new group of metal-sensing regulatory RNA elements in addition to the characterized magnesium riboswitches (5, 9).

MntS could function as a manganese chaperone. We observed that cells lacking MntS fail to properly repress mntH expression when exposed to manganese (Fig. 6). This finding could suggest that MntS directly modifies MntR activity, either as a transcription corepressor or by activating DNA binding of MntR by facilitating its association with manganese. We currently favor the role as a facilitator. MntS could act in a similar manner for the Fur repressor. Alternatively, MntS could affect mntH repression by altering cellular manganese pools and global availability, which would perturb MntR and/or Fur activity. The MntS small protein could also affect MntH activity itself, thereby affecting the amount of manganese imported into cells.

In principle, the mntS transcript could function as regulatory RNA like the SgrS RNA does, which regulates target mRNAs by base pairing in addition to serving as an mRNA (46). The reciprocal expression of mntP and mntS suggest negative regulation of mntP by the mntS transcript. However, we did not observe a change in basal or induced levels of mntP in strains lacking the MntS open reading frame or the entire mntS transcript (data not shown). In conjunction with our phenotypic data for overproduction of the MntS protein, we conclude that the mntS transcript affects manganese homeostasis through the MntS small protein. The mntS transcript was not found to comiplimentate with the RNA chaperone Hfq (48), suggesting that it does not function by base pairing. Moreover, regions of potential base pairing, which by analogy with SgrS would be outside the MntS coding region, are poorly conserved (Fig. 1B). Therefore, although a formal possibility for any mRNA, we do not expect that the mntS transcript acts as a regulatory RNA, at least for manganese homeostasis.

Instead, given that MntS is most abundant when manganese is limiting and that overproduction of MntS potentiates man-
ganese toxicity, MntS may function as a manganese chaperone to make manganese more available by delivering it to the necessary cellular locations when the ion is limiting. This is an attractive possibility, because manganese is a relatively uncompetitive metal and is likely to be outcompeted by other metals for insertion into metal binding sites in proteins (47). Therefore, manganese probably requires additional specificity-determining steps, such as delivery by a metallochaperone, to be acquired with high fidelity. In this model, overproduction of MntS under conditions of manganese excess could cause aberrant missetallation of proteins, inactivating them and causing sensitivity to manganese (Fig. 5A). MntS possesses several conserved Asp, Glu, and His amino acids that could coordinate manganese, as well as two conserved Cys residues. Although manganese is considered to prefer chelation by carboxylate moieties, emerging findings demonstrate that some manganese-dependent enzymes use Cys residues to coordinate manganese ions (J. Imlay, personal communication). However, a role for MntS in binding to other metals or in redox reactions cannot be excluded at this time.

To date, only a few enzymes are known to preferentially use manganese over other metals in vivo (27); however, this topic is only beginning to be systematically investigated. Considering that cells maintain manganese at concentrations below most other metals (12), how proteins specifically obtain manganese is a problem of significant interest. In addition, little is known about the competition between manganese and other metals in vivo. Understanding the role for MntS in binding to other metals or in redox reactions cannot be excluded at this time.

REFERENCES


