Metallobiology of Tuberculosis

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ABSTRACT Transition metals are essential constituents of all living organisms, playing crucial structural and catalytic parts in many enzymes and transcription factors. However, transition metals can also be toxic when present in excess. Their uptake and efflux rates must therefore be carefully controlled by biological systems. In this chapter, we summarize the current knowledge about uptake and efflux systems in Mycobacterium tuberculosis for mainly three of these metals, namely iron, zinc, and copper. We also propose questions for future research in the field of metallobiology of host-pathogen interactions in tuberculosis.

THE BATTLE FOR IRON
Iron is absolutely required for the life of most organisms, including mycobacteria. Iron is incorporated into proteins, either as a mono- or binuclear species or as part of heme groups or iron-sulfur clusters. Iron undergoes reversible changes in its oxidation state, oscillating between the oxidized ferric (Fe³⁺) and the reduced ferrous (Fe²⁺) forms. In addition, depending on the local ligand environment, iron-containing compounds exhibit a wide range of oxidation-reduction potentials. These unique properties make this metal a very versatile prosthetic component as a biocatalyst and electro-carrier in essential cellular pathways including respiration, the trichloroacetic acid (TCA) cycle, oxygen transport, gene regulation, defense against oxidative stress, and DNA biosynthesis (1).

Iron is the fourth most plentiful element in the earth’s crust. Before oxygenic photosynthesis it was found in its soluble ferrous form (solubility 0.1 M at pH 7.0); however, introduction of oxygen into the atmosphere caused a switch to the ferric form, which is insoluble as ferric hydroxide. In consequence, free iron became extremely scarce (solubility 10⁻¹⁸ M at pH 7.0). In host tissues, the concentration of this metal is lowered even further as Fe(III) is sequestered by iron binding proteins such as transferrin, lactoferritin, and ferritin (2, 3). In addition, the host produces proteins that either efflux iron from intracellular microbial compartments (NRAMP1) or bind heme and hemoglobin (e.g., hemopexin and haptoglobin) and reduce the availability of heme as an iron source (2, 4, 5). Thus, iron starvation in the host is a serious threat for infecting bacteria and has been recognized as such for decades (3). Pathogens are able to survive and multiply in the host in part because they have evolved numerous and often redundant high-affinity iron acquisition mechanisms, including (i) acquisition of iron directly from host iron binding proteins (e.g., transferrin and lactoferrin) by using receptor-mediated transport systems, (ii) uptake and utilization of heme, (iii) solubilization of ferric oxides by reduction of ferric iron and transport of soluble ferrous iron, and (iv) production of ferric iron chelators (siderophores) in conjunction with siderophore-based transport systems. Mycobacterium tuberculosis obtains iron by producing siderophores, and it also has the capacity to utilize heme as an iron source in a siderophore-independent manner.

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Siderophore-Based Iron Acquisition in *M. tuberculosis*

*M. tuberculosis* produces mycobactin, a cell-associated, lipophilic siderophore, and a soluble amphiphilic variant of it named carboxymycobactin (6). *M. tuberculosis* does not produce or utilize exochelin, the peptidic siderophore synthesized by nonpathogenic mycobacteria such as *Mycobacterium smegmatis*. Mycobactin and carboxymycobactin are composed of a hydroxyaromatic acid, an oxazoline moiety, a β-hydroxy acid, and two ε-N-hydroxylysines (Fig. 1). Genomic and biochemical analysis indicates that the *mbt*-1 gene cluster (*mbt-IABCDEFG*) encodes the proteins necessary for the assembly of the siderophore core: Mbtl for salicylate synthesis; the hybrid nonribosomal peptide synthase/polyketide synthase (MbtA-F); and the L-Lys hydroxylase (MbtG) (7–9). Mycobactin and carboxymycobactin differ mainly in an acyl group attached to the central L-Lys residue. Mycobactin has a long fatty acyl chain (10 to 21 carbons) that is capped by a methyl group, whereas carboxymycobactin has a shorter acyl chain (2 to 9 carbons) that is capped with either a carboxylate or methyl ester (10, 11). The presence of the long acyl chain makes mycobactin very hydrophobic and ensures retention within or in close proximity to the cytoplasmic membrane. Mycobactin may mediate uptake of iron donated by amphiphilic molecules that can penetrate the cell wall, for instance, carboxymycobactin and acinetoferrin (12, 13). Carboxymycobactin, being more polar than mycobactin, is water soluble and exported to the extracellular medium.

The process of carboxymycobactin export seems to be coupled—in an unknown way—to its synthesis and depends on two redundant systems composed of the MmpL4 and MmpL5 transporters and their associated proteins, MmpS4 and MmpS5 (14). These transport systems are postulated to mediate export of carboxymycobactin into the periplasm. However, the hypothetical outer membrane protein that mediates release of carboxymycobactin into the extracellular medium is unknown (Fig. 2). *M. tuberculosis* mutants unable to synthesize or export siderophores are drastically attenuated in a mouse model of tuberculosis infection, underscoring the importance of efficient iron acquisition for propagation of *M. tuberculosis* (14).

In the extracellular environment, carboxymycobactin avidly captures ferric iron. Ferric-carboxymycobactin can slowly transfer iron to mycobactin (12) or deliver this metal via the iron-regulated transporter, IrtAB. IrtAB is an ABC-type transporter synthesized in cells experiencing iron limitation and is necessary for Fe3+-carboxymycobactin uptake. IrtAB mutants are iron deficient and fail to replicate normally in macrophages and in mice (15). Interestingly, the amino-terminal domain of the IrtA protein is located in the cytoplasm and has a functional flavin adenine dinucleotide (FAD) binding motif. Mutations that prevent FAD binding affect assimilation of iron imported by IrtAB. Since a common mechanism to dissociate iron-siderophore complexes is reduction of ferric iron to ferrous iron by cytoplasmic flavin reductases, it is possible that the amino-terminal domain of IrtA functions as a FAD-dependent ferric...

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**FIGURE 1** Carboxymycobactin and mycobactin share a common core structure but differ in the length of the alkyl substitution that determines their polarity and hence solubility. The groups involved in binding of Fe(III) are indicated in bold. doi:10.1128/microbiolspec.MGM2-0012-2013.f1
reductase that mediates the release of ferrous iron from imported Fe\textsuperscript{3+}-carboxymyco- 

bacitin (cMB) and mycobactin (MB). MB remains cell associated, although the precise 
location is not clear. cMB is secreted by a process dependent on the membrane proteins 
MmpL4 and MmpL5 and requiring the MmpS4 and MmpS5 membrane-associated proteins 
that function together with their cognate MmpL proteins. Proteins that mediate export of 
cMB across the outer membrane remain to be discovered. Once secreted, cMB chelates 
Fe\textsuperscript{3+} and possibly requires an outer membrane and periplasmic protein to reach the IrtAB 
importer in the inner membrane. In the cytosol, the FAD binding domain of IrtA may reduce 
ferric iron to ferrous iron and dissociate the iron-siderophore complex. Released ferrous 
iron can be utilized and stored in ferritins. Excess iron binds to the regulator IdeR and 
activates its DNA binding activity. Binding of IdeR to the promoters of siderophore syn-
thesis, secretion, and transport represses the expression of those genes, turning off iron 
uptake. Meanwhile, IdeR-Fe\textsuperscript{2+} binding to the promoters of ferritins (ferritin and 
bacterioferritin) turns on iron storage, thereby preventing iron-mediated toxicity and 
maintaining iron homeostasis. 


![Diagram of Fe\textsuperscript{3+} and cMB transport](image)

**FIGURE 2** When experiencing iron limitation, *M. tuberculosis* produces carboxy-
mycobactin (cMB) and mycobactin (MB). MB remains cell associated, although the precise 
location is not clear. cMB is secreted by a process dependent on the membrane proteins 
MmpL4 and MmpL5 and requiring the MmpS4 and MmpS5 membrane-associated proteins 
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carboxymycobactin (17, 18). These findings suggest that directly or indirectly, components of the Esx-3 system may contribute to Fe³⁺-carboxymycobactin uptake.

**Heme Utilization**

Many pathogens have evolved strategies to obtain iron from heme, which is the most abundant source of iron in mammals. Bacteria can obtain heme using outer membrane receptors and periplasmic binding protein-dependent ABC transporters specific for heme, or they synthesize and secrete specialized proteins (hemophores) able to sequester heme and deliver it to a specific outer membrane receptor. *M. tuberculosis* is able to obtain iron from heme in the absence of siderophores (19). A genetic region encoding a secreted heme binding protein (hemophore) and two membrane transporters is necessary for normal iron acquisition from heme and hemo-globin (20). Once internalized, heme has to be degraded to release iron. This function is usually performed by heme oxygenases that degrade heme into iron, a tetrapyrrole product, and carbon monoxide (CO). In *M. tuberculosis* this role might be performed by the enzyme MhuD, a homolog of the *Staphylococcus aureus* heme oxygenases IsdG and IsdI. MhuD degrades heme in a unique way: it releases iron and a tetrapyrrrole product named mycobilin but without CO generation (21). Presently, the role of heme uptake in the pathogenesis of *M. tuberculosis* is unknown. Studies in animal models will determine the relevance of heme utilization in *M. tuberculosis* virulence.

**Iron Storage**

The synthesis of iron storage proteins (ferritins) is central to iron homeostasis in most aerobic organisms and necessary for the virulence of many pathogens. Ferritin subunits form a hollow sphere where up to 4,500 atoms of iron can be sequestered as mineral, after being oxidized to Fe³⁺ at a ferroxidase center (22). Some bacteria and fungi synthesize ferritin-like proteins containing heme b known as bacterioferritins. *M. tuberculosis* has a bacterioferritin (BfrA) and a ferritin (BfrB). The crystal structure of these proteins shows the typical architecture of the ferritin superfamily of a cage-like hollow shell formed by 24 monomers with the characteristic fold of a four-helical bundle containing the ferroxidase catalytic center, and in bacterioferritin a heme group in each subunit-pair interface (23, 24). Analyses of single deletion mutants of *M. tuberculosis* showed that BfrA and BfrB are not functionally redundant. Deletion of bfrB drastically altered iron homeostasis, whereas no obvious defects were detected in a bfrA-deleted mutant (25). Iron stored by BfrB seems to be *M. tuberculosis*’s preferred reserve to overcome iron deficiency. In addition, *M. tuberculosis* lacking BfrB is highly sensitive to peroxide- and antibiotic-generated oxidative stress when cultured in iron-rich media. This indicates that BfrB is required to prevent excess free iron from catalyzing the generation of toxic reactive oxygen species (25). The significance of proper iron storage in the pathogenesis of *M. tuberculosis* has been demonstrated in animal models of infection. A mutant lacking *bfrB* is unable to persist in the lungs of mice and establish infection in the liver (25). Furthermore, a double *bfrA*/bfrB mutant is strongly attenuated in a guinea pig model of tuberculosis infection (26).

In addition to BfrA and BfrB, *M. tuberculosis* possesses a histone-like DNA binding protein (MDP1) that captures iron and also has ferroxidase activity. MDP1 may protect DNA by preventing the local generation of reactive oxygen radicals (27).

**Regulation of Iron Metabolism**

Iron can be very toxic because it catalyzes the generation of reactive oxygen species from normal products of aerobic respiration via the Harber-Weiss and Fenton reactions. Reactive oxygen species can damage most cellular components including DNA, lipids, and proteins. For this reason, aerobic organisms must tightly control intracellular iron levels. In bacteria, this control is generally achieved by regulating the uptake, utilization, and storage of this metal. Like other prokaryotes, *M. tuberculosis* regulates iron metabolism at the level of gene transcription. It induces the expression of iron uptake genes under iron deficiency and upregulates iron storage and oxidative stress defense genes when iron is readily available (28). *M. tuberculosis* achieves the delicate balance between the requirement for iron and its toxicity through the function of the iron-dependent regulator IdeR. IdeR is a metal and DNA binding protein, closely related to the *Corynebacterium diphtheriae* regulator of iron metabolism and toxin production DtxR (29). The structure of IdeR revealed two metal binding sites and three distinct functional domains: the amino-terminal containing a helix-turn-helix DNA binding motif, a dimerization domain that also bears toxic balance between the requirement for iron and its toxicity through the function of the iron-dependent regulator IdeR. IdeR is a metal and DNA binding protein, closely related to the *Corynebacterium diphtheriae* regulator of iron metabolism and toxin production DtxR (29). The structure of IdeR revealed two metal binding sites and three distinct functional domains: the amino-terminal containing a helix-turn-helix DNA binding motif, a dimerization domain that also bears most of the metal binding residues, and the carboxy-terminal domain characterized by adopting an SH3 (Src homology domain 3)–like folding, suggesting possible interactions with other proteins (30). Metal binding stabilizes dimer formation (31) and activates DNA binding. As two dimers, IdeR binds to both faces of the DNA at a unique 19-bp inverted repeat sequence, the
“iron box” (TTAGGTAGGCTAACCTAA), present in the promoter of iron-regulated genes, thereby modulating their transcription (32). Disruption of the ideR gene in M. tuberculosis is only possible in the presence of a second copy of the gene or when suppressor mutations arise. This indicates that IdeR is essential in M. tuberculosis (28). Approximately 150 genes respond to changes in iron availability in M. tuberculosis. IdeR controls the expression of about one-third of those genes including the siderophore synthesis and export genes, the siderophore transporter encoding genes irtA and irtB, genes in the esx-3 cluster, and the iron storage genes bfrA and bfrB (28, 32). IdeR and Fe^{2+} turn off iron acquisition and turn on iron storage (Fig. 2).

These opposite effects of IdeR as a repressor of iron uptake and an activator of iron storage can be understood by considering the position of the iron box in repressed and activated promoters. Iron boxes on IdeR-repressed genes overlap the −10 region or the transcriptional start site; consequently, binding of IdeR to the iron box blocks access of the RNA polymerase and inhibits transcription of those genes. In the promoters of bfrA and bfrB, tandem iron boxes are located farther upstream (100 to 106 bp) from the transcriptional start site, suggesting a mechanism of activation by which IdeR-Fe^{2+} bound to these sites enhances access of the RNA polymerase to the promoter and initiation of transcription. In view of the strong attenuation of iron storage mutants in vivo (25), it is likely that IdeR-mediated activation of iron storage is essential for growth of M. tuberculosis during infection.

**ZINC AND COPPER: NEVER TOO LITTLE OR TOO MUCH**

Zinc and copper play vital functions in biological systems. The chemical properties of zinc, e.g., its Lewis acidity, coordination geometry, and rapid ligand exchange, allow it to form stable complexes with enzymes and proteins, where it functions in catalysis or as a structural factor. The majority of zinc-containing enzymes catalyze hydrolysis or related transfer reactions, some of which are essential for cell viability. The number of zinc-containing proteins identified in mycobacteria has increased significantly as more protein structures are resolved. Zinc is part of M. tuberculosis zinc-metallopeptidases (33, 34), carbonic anhydrase (35), fructose biphosphate aldolase Fba (36), the helicase RqlH (37), the cytidine deaminase Cda (38), the MshC ligase involved in mycothiol biosynthesis (39), the 2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase IspF (40), the 2-isopropylmalate synthase LeuA involved in leucine biosynthesis (41), the superoxide dismutase (SOD) SodC (42, 43), the Esx-3 substrate EsxG-EsxH complex (44), the inositol-1-phosphate synthase (45), the RecA intein (46), and several more.

Because of its fast interconversion of Cu^{+} and Cu^{2+}, copper is involved in several essential biochemical processes, such as oxygen-dependent electron transport reactions. In M. tuberculosis, at least two enzymes require copper as a cofactor, namely the SOD SodC (42, 43) and the cytochrome c oxidase subunits CtaC and CtaD.

Bioavailable levels of zinc are sufficiently low that most microbes have evolved high-affinity transport systems to capture this metal. Bacterial zinc transporters are generally ABC transporters consisting of a periplasmic binding protein, a membrane permease, and an ATPase. The periplasmic binding protein, which usually has a central His-, Asp-, and Glu-rich region, seems to allow specificity for zinc over manganese and other cations (47). Proteins involved in zinc import in mycobacteria have yet to be discovered. Regarding copper, as in most bacterial species, uptake systems for this metal have not been identified in M. tuberculosis.

The metallobiology of zinc and copper in M. tuberculosis recently provided insights into novel host defense mechanisms against bacterial infection involving intoxication by metal ions. To resist potential intoxication by metal ions, microbes express a range of metal efflux pumps and transporters belonging to three main families: heavy metal efflux members of the resistance–nodulation–cell division superfamily (HME-RND), the cation diffusion facilitator family, and the P-type ATPase family (48). A set of recent studies strikingly reported that some of these efflux systems are required for microbial virulence in various bacterial species, including M. tuberculosis, in order to resist newly described immune mechanisms relying on metal poisoning of microbes inside host cells.

In the M. tuberculosis genome, no putative heavy metal efflux system of the HME-RND family has been detected, while one putative cation diffusion facilitator transporter (Rv2025c) and 12 putative P-type ATPase members (CtpA-J, CtpV, and KdpB) are present (48, 49). The exact substrate specificity of these transporters is not known and is mostly inferred from indirect evidence such as similarity to known transporters and the presence of conserved metal binding motifs. For instance, the transcriptional regulator CmtR/Rv1994c, present in operons with the P-ATPase CtpG, responds to cadmium and lead (50) to alleviate ctpG transcriptional repression, suggesting that CtpG can efflux these two...
heavy metal cations; similarly, the ability of NmtR/Rv3744 to respond to nickel and cobalt and to bind the promoter region of the neighbor gene ctpJ/nmtA/Rv3743c to repress its expression in metal-free conditions (51) again suggests that CtpJ may efflux nickel and cobalt. Finally, the recent findings that \( M. \) \( \text{tuberculosis} \) mutants inactivated in ctpV and ctpC are highly sensitive to copper and zinc, respectively (52, 53), strongly suggest that these two P-ATPases may transport these metal ions, but again this is not a proof of their metal selectivity. Biochemical characterization of these transporters in recombinant biological systems and in reconstituted liposomal fractions will be required in order to understand their function.

In this context, a striking feature of three P-ATPase members in \( M. \) \( \text{tuberculosis} \), namely CtpC, CtpG, and CtpV, is the presence of a putative metallochaperone-encoding gene, namely and respectively, Rv3269, Rv1993c, and Rv0968, upstream of the P-ATPase-encoding genes. The P-ATPase- and metallochaperone-encoding genes seem to be expressed in the operon. The function of these small proteins, predicted to be membrane bound and exposing putative metal binding motifs (e.g., DDGHDH in Rv0968) in their C-terminal cytoplasmic part, is not known; however, it is tempting to speculate that they may play a key part in metal selectivity and the transport mechanism of their cognate P-ATPase, as recently demonstrated for a similar transport system in \( \text{Streptococcus pneumoniae} \) (54).

A role for P-ATPase-mediated metal detoxification in \( M. \) \( \text{tuberculosis} \) has been recently suggested by several independent reports. In particular, \( M. \) \( \text{tuberculosis} \) mutants inactivated in the P-ATPase-encoding genes ctpV and ctpC were shown to be impaired in their ability to proliferate in model animals and/or host macrophages (52, 53). In a guinea pig model, Ward et al. reported that lung colonization by \( M. \) \( \text{tuberculosis} \) \( \Delta \)ctpV was reduced by \( \approx 1 \log_{10} \) 3 weeks after inoculation, compared to the wild-type strain, and full virulence of the mutant was restored upon genetic complementation (53). The same authors reported a similar observation in mice, where the survival rate of animals infected with the mutant strain was increased by 16 weeks compared to those infected with the wild-type strain, although unlike in guinea pigs, no CFU difference was noticed in the mouse lungs. In both animal models, lung granulomatous inflammation was severely reduced in animals infected with the \( \Delta \)ctpV mutant compared to the wild-type strain.

Although no direct demonstration has been provided yet regarding the metal selectivity of CtpV, it is most likely that this P-ATPase effluxes copper, because (i) the ctpV gene is induced by copper (55, 56), (ii) the CtpV protein contains typical motifs of the P1B1 family of copper-transporting P-ATPases (Table 1), (iii) the ctpV

### TABLE 1 P-ATPases in \( M. \) \( \text{tuberculosis} \)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Group</th>
<th>Predicted substrate(s)</th>
<th>Comments</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctpA/Rv0092</td>
<td>1B1</td>
<td>Cu(^+)/Ag(^+)</td>
<td>N-terminal CxxC; C-terminal MxxSS</td>
<td>53</td>
</tr>
<tr>
<td>ctpB/Rv0103c</td>
<td>1B1</td>
<td>Cu(^+)/Ag(^+)</td>
<td>N-terminal CxxC; C-terminal MxxSS</td>
<td></td>
</tr>
<tr>
<td>ctpC/Rv3270</td>
<td>1B?</td>
<td>Zn(^{2+}); possibly others</td>
<td>Putative metallochaperone Rv3269; ( M. ) ( \text{tuberculosis} ) mutant sensitive to zinc</td>
<td>84</td>
</tr>
<tr>
<td>ctpD/Rv1469</td>
<td>1B4</td>
<td>Co(^{2+})</td>
<td>C-terminal HEGxT; ( M. ) ( \text{smegmatis} ) homologue transports Co(^{2+})</td>
<td></td>
</tr>
<tr>
<td>ctpE</td>
<td>2A-like</td>
<td>Unknown</td>
<td>PEGL(P/V) motif found in calcium-transporting P-ATPases, such as SERCA</td>
<td></td>
</tr>
<tr>
<td>ctpF</td>
<td>2A</td>
<td>Ca(^{2+})</td>
<td>PEGL(P/V) and Tm6-LWxNxxxd motifs found in calcium-transporting P-ATPases, such as SERCA</td>
<td>81</td>
</tr>
<tr>
<td>ctpG/Rv1992c</td>
<td>1B?</td>
<td>Possibly Cd(^{2+})/Pb(^{2+}) and others</td>
<td>Putative metallochaperone Rv1993c, repressed by Rv1994c/NmtR, unless Cd(^{2+}) or Pb(^{2+}) is present</td>
<td></td>
</tr>
<tr>
<td>ctpH</td>
<td>2A-like</td>
<td>Unknown</td>
<td>Large N-terminal membrane-spanning domain; Tm6-PEGL(P/V) motif found in calcium-transporting P-ATPases, such as SERCA</td>
<td></td>
</tr>
<tr>
<td>ctpI</td>
<td>2A-like</td>
<td>Unknown</td>
<td>Large N-terminal membrane-spanning domain; Tm6-PEGL(P/V) motif found in calcium-transporting P-ATPases, such as SERCA</td>
<td></td>
</tr>
<tr>
<td>ctpJ/Rv3743c</td>
<td>1B4</td>
<td>Co(^{2+})</td>
<td>C-terminal HEGxT; repressed by Rv3744/NmtR, unless Ni(^{2+}) or Co(^{2+}) is present</td>
<td>51</td>
</tr>
<tr>
<td>ctpV/Rv0969</td>
<td>1B1</td>
<td>Cu(^{+})</td>
<td>C-terminal MxxSS; ( M. ) ( \text{tuberculosis} ) mutant sensitive to copper; putative metallochaperone Rv0968; in operon with copper-responsive regulator csoR/Rv0967</td>
<td>53, 56</td>
</tr>
<tr>
<td>kdpB/Rv1030</td>
<td>1A</td>
<td>K(^+)</td>
<td>Homologous to many KdpB potassium transporters</td>
<td></td>
</tr>
</tbody>
</table>
gene is encoded in operons with the copper-responsive transcriptional repressor CsoR (57), and most importantly, (iv) the ΔtcpV mutant is highly sensitive to copper in vitro (53). These results suggesting that M. tuberculosis faces copper intoxication in vivo during infection were further strengthened by a report that showed that the outer membrane channel protein Rv1698/MctB is also required for both copper detoxification in vitro and for full virulence in vivo in guinea pigs (58). It was thus proposed that copper accumulation inside the mycobacterial phagosome may account for the phenotype of the ΔtcpV and ΔmctB mutants in vivo (59–61).

Phagosomal intoxication by copper has been suggested in other settings; in particular, an elegant study conducted in Escherichia coli–infected macrophages reported that copper enhances intracellular bacterial killing inside macrophages and that an E. coli mutant inactivated in the copper efflux P-ATPase CopA is killed faster in macrophages than its wild-type counterpart, unless the eukaryotic copper transporter ATP7A, which traffics to phago-lysosomes, is silenced through interference RNA (iRNA) (62). Although copper accumulation in the bacterial vacuole was not directly evidenced, this elegant study suggested for the first time that copper is an important mediator of microbial killing by immune cells and provided a mechanistic explanation for this phenomenon.

Regarding CtpC, we reported that genetic inactivation of this P-ATPase dramatically increases M. tuberculosis sensitivity to Zn^{2+}, which strongly suggested that CtpC might be involved in zinc efflux (52). However, a recent report suggested that CtpC may transport Mn^{2+} over Zn^{2+} and that the hypersensitivity of the ctpC mutant to zinc may be due to an increased sensitivity to oxidative stress following impaired Mn^{2+} loading of the SOD SodA and possibly other detoxification systems (63). Inside macrophages, we showed that zinc accumulates within E. coli– or M. tuberculosis–containing phagosomes and that bacterial strains impaired in resistance to zinc (a ΔntA mutant in E. coli or a ΔctpC mutant in M. tuberculosis) are impaired in intracellular survival. In vivo attenuation of the M. tuberculosis ΔctpC mutant has yet to be clearly established (52, 63).

The requirement of P-ATPase-mediated copper resistance systems in bacterial virulence has been documented in several bacterial species, including Listeria monocytogenes (64), Pseudomonas aeruginosa (65), S. pneumoniae (66), and Salmonella typhimurium (67). Several mechanisms have been proposed to explain copper ion toxicity. These mechanisms include Fenton chemistry and generation of hydroxyl radicals (although this was challenged by data showing that there is no accumulation of hydroxyl radicals in copper-exposed E. coli [68]); degradation of iron-sulfur clusters in enzymes (69); and replacement of other metal ion cofactors, such as zinc ions, in proteins. The exact mechanism(s) of copper toxicity in M. tuberculosis remains to be identified.

The mechanism(s) of zinc ion toxicity may also include inactivation of iron-sulfur clusters (70) and inhibition of manganese uptake through transport competition in the bacterial periplasm (71). It was shown recently that P-ATPase-mediated copper export is required for the copper supply to periplasmic Cu,Zn-SOD and resistance to oxidative stress in Salmonella enterica (72). Whether copper and zinc export through CtpV, CtpC, and possibly other P-ATPases contributes to activation of the periplasmic Cu,Zn-SOD SodC in M. tuberculosis remains to be evaluated.

In summary, it is clear that M. tuberculosis uses the P-ATPases CtpC and CtpV to thrive inside macrophages and resist poisoning by Zn^{2+} and Cu^{2+}. The function of the other M. tuberculosis P-ATPases, and their possible implication in mycobacterial virulence, remain to be understood. Equally important will be to understand the function of the putative metallochaperones associated with CtpC, CtpG, and CtpV.

### Regulation of Metal Uptake

As stated above, although necessary, zinc and other metal ions can also be toxic if present at too high a concentration. For instance, zinc may interact with thiols or compete with other metals for protein binding, blocking essential reactions in the cells. Therefore, the quantity of zinc inside the cells is carefully regulated, usually by calibrating uptake and export. The genome of M. tuberculosis contains two genes encoding transcriptional regulators of the Fur family, FurA and FurB. Structural and functional characterization of FurB revealed it to be a Zn^{2+}-dependent repressor; hence, it has been renamed Zur (zinc uptake regulator) (73–75). Genes repressed by Zur-Zn^{2+} include the gene cluster encoding the Esx-3 secretion system, several ribosomal proteins, a protein similar to the Bacillus subtilis low-affinity zinc transporter YciC, and components of a putative ABC-type Zn^{2+}/Mn^{2+} transport system (74). Disruption of the zur gene did not affect the ability of M. tuberculosis to replicate in mice, suggesting that constitutive expression of Zur-regulated genes is not detrimental for M. tuberculosis in this model of infection (74).
The importance of sensing metal deficiency or excess is reflected in the multiple families of metalloregulatory proteins characterized in bacteria. These include Fur, DtxR, MerR, SmtB/AnsR, CsoR, CopY, and NikR. In general, these proteins are transcriptional regulators that sense specific metal ions via direct coordination. The DtxR, Fur, and NikR family proteins primarily regulate genes required for metal uptake, whereas members of the other families regulate mainly metal efflux. Fur was first described as an iron-responsive repressor of iron transport in E. coli. Since then, numerous studies have revealed functional specialization within the Fur family and a great diversity in metal selectivity and biological function. The Fur family includes sensors of iron (Fur), zinc (Zur), manganese (Mur), and nickel (Nur). Some members of the family use metal-catalyzed redox reactions to sense peroxide-mediated stress (Per) or heme (Irr).

*M. tuberculosis* has two Fur-like proteins, namely Zur (described above) and FurA. The FurA-encoding gene is located immediately upstream of *katG*, the gene encoding a catalase-peroxidase, a major virulence factor that also activates the prodrug isoniazid. *furA* and *katG* are cotranscribed from a common promoter upstream of *furA*. FurA auto-represses its expression and the expression of *katG* by binding to a unique sequence upstream of *furA* (76–78). FurA seems to have a very specialized biological role, as no other genes regulated by FurA have been identified to date.

Two regulators of the DtxR family are present in *M. tuberculosis*: the iron-dependent regulator IdeR (described above) and SirR (for staphylococcal iron-regulated repressor). In *Staphylococcus epidermidis*, SirR, in complex with Fe$^{2+}$ or Mn$^{2+}$, binds to a unique sequence in the promoter of an operon encoding for a putative iron transporter (79). The biological role of the SirR homologue in *M. tuberculosis*, however, has not been determined.

Other metalloregulators characterized in *M. tuberculosis* include the Ni(II)/Co(II)-specific repressors NmtR (51) and KmtR (80), the copper sensors CsoR (57) and RicR (55), the Cd(II)/Pb(II) sensor CmtR (81), and the Zn(II)-responsive regulator encoded by the gene Rv2358 (82). In general, they regulate the transcription of membrane transporters that mediate cytoplasmic efflux of potentially toxic metals, as mentioned above.

**FUTURE DIRECTIONS**

Much remains to be understood regarding the mechanisms of transition metal uptake/efflux systems in *M. tuberculosis*, their regulation, and the biological impact of selective metal ion enrichment or depletion encountered in the mycobacterial phagosome inside host macrophages (59, 83). As mentioned above, the zinc and copper uptake systems still have to be identified in *M. tuberculosis*. Identification of the remaining components of the iron acquisition apparatus and a better understanding of the mechanisms that control iron sorting and assimilation in *M. tuberculosis* will reveal new possibilities of intervention. For instance, ways to starve *M. tuberculosis* for iron or, alternatively, get it to intoxicate itself by corrupting its iron-sensing mechanisms may help develop novel treatments. Future work should also aim at deciphering the exact metal specificity and biological function of the many *M. tuberculosis* P-ATPases, and the use innate immune cells make of metal ion withdrawal or intoxication to contain mycobacterial infection.

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