A novel P₁β-type Mn²⁺ transporting ATPase is required for secreted protein metallation in mycobacteria

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*Running title: CtpC a mycobacterial Mn²⁺-ATPase

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Background: CtpC is an uncommon metal transport ATPase required for Mycobacterium tuberculosis virulence.

Results: CtpC shows Mn²⁺-ATPase activity. Mutations in ctpC alter Mn²⁺ homeostasis, increases sensitivity to redox stress and decrease Mn-Sod activity.

Conclusion: CtpC is a Mn²⁺ transport ATPase required for homeostasis and the assembly of secreted metalloproteins in mycobacterium.

Significance: CtpC provides a novel mechanism for Mn²⁺ metallation of secreted proteins.

SUMMARY

Transition metals are central for bacterial virulence and host defense. P₁β-ATPases are responsible for cytoplasmic metal efflux, and play roles either in limiting cytosolic metal concentrations or in the maturation of secreted metalloproteins. The P₁β-ATPase, CtpC, is required for Mycobacterium tuberculosis survival in a mouse model (Sassetti and Rubin (2003) Proc. Nat. Acad. Sci. 100, 12989-12994). CtpC prevents Zn²⁺ toxicity, suggesting a role in Zn²⁺ export from the cytosol (Botella, et al., (2011) Cell Host Microbe 10, 248-259).

However, key metal coordinating residues and overall structure of CtpC are distinct from Zn²⁺-ATPases. We found that isolated CtpC has metal dependent ATPase activity with a strong preference for Mn²⁺ over Zn²⁺. In vivo, CtpC is unable to complement Escherichia coli lacking a functional Zn²⁺-ATPase. Deletion of M. tuberculosis or M. smegmatis ctpC leads to cytosolic Mn²⁺ accumulation but no alterations in other metals levels. While ctpC-deficient M. tuberculosis is sensitive to extracellular Zn²⁺, the M. smegmatis mutant is not. Both ctpC mutants are sensitive to oxidative stress, which might explain the Zn²⁺-sensitive phenotype of the M. tuberculosis ctpC mutant. CtpC is a high affinity/slow turnover ATPase, suggesting a role in protein metallation. Consistent with this hypothesis, mutation of CtpC leads to a decrease of Mn²⁺-bound to secreted proteins and of the activity of secreted Fe/Mn-superoxide dismutase, particularly in M. smegmatis. Alterations in the assembly of metalloenzymes involved in redox stress response might explain the sensitivity of M. tuberculosis ctpC mutants to oxidative stress, growth and persistence defects in mice infection models.

Transition metals are essential for life, however at high concentrations can become toxic.
due to either adventitious metal binding to various biomolecules, or the promotion of oxidative stress through Fenton chemistry (1,2). To prevent metal toxicity, chaperone and chelating molecules tightly bind free ion species and transmembrane transporters prevent excess accumulation in the cytosol. Together, these mechanisms maintain the aqueous milieu essentially free of uncomplexed metals (3-5). As a consequence of tightly regulated metal homeostasis, metalloproteins acquire the necessary cofactors through specific protein-protein interactions (4).

The involvement of transition metals in host-pathogen interaction is highlighted by the requirement of various transition metal transporters, as well as transition metal-responsive transcriptional regulators, for bacterial virulence (3,6). Intracellular pathogens, such as *Mycobacterium tuberculosis*, must cope with transition metal starvation or excess stress during infection. For example, the natural resistance-associated macrophage protein 1 (Nramp1), a divalent cation-proton antiporter, participates in bacterial killing by driving Fe$^{2+}$ efflux from the phagosome and starving the enclosed bacteria for this essential nutrient (7). Whether or not transition metals other than Fe$^{2+}$ are depleted in the phagosome after infection is unclear (8,9). In addition to the transition metal starvation model, it has been proposed that transporter recruitment to the phagosomal membrane may generate stress by increasing the concentration of free transition metal such as Cu$^{+}$ and Zn$^{2+}$ (9-11). In particular, there is evidence that Zn$^{2+}$ accumulation during infection hampers the growth of intracellular pathogens (11). Supporting this, expression of the lysosome-associated Zn$^{2+}$ transporter ZIP8 in IFNγ-stimulated macrophages and T-cells has been reported (12,13).

Transition metals also appear to be relevant for bacterial virulence through their participation in redox detoxification by metalloenzymes. For instance, *M. tuberculosis* survives in a highly oxidative environment by means of intracellular and secreted superoxide dismutases (Sod)$^3$ that participate in ROS and RNS detoxification mechanisms (14-16). *M. tuberculosis* SodA, is a secreted Fe$^{2+}$/Mn$^{2+}$ dependent SOD protein, but under *in vitro* growth conditions contains Fe$^{2+}$ as a primary cofactor (14,16,17). SodA is exported in an unfolded state via a Sec-dependent mechanism (18) likely acquiring its metal cofactor in the extra-cytoplasmic milieu.

Heavy metal transport P$_{1B}$-ATPases$^5$ drive the efflux of a range of cytoplasmic metal ions such as Cu$^{+}$, Zn$^{2+}$ and Co$^{2+}$ using the energy of ATP hydrolysis and are involved in maintaining cellular metal quotas (3,19). The metal specificity of most P$_{1B}$-ATPase can be predicted based on the conserved signature sequences present in transmembrane segments (TM) TM6, TM7, and TM8 (3,19-23). There are seven transition metal transporting P$_{1B}$-ATPases in *M. tuberculosis* (20). Their signature transmembrane metal binding sites (TM-MBS) indicate that three (CtpA, CtpB and CtpV) are Cu$^{+}$-ATPases and two others are likely Co$^{2+}$-ATPases (CtpD and CtpJ), while CtpC and CtpG appear to be novel transition metal transporters. Large-scale genetic screens have predicted that ctpC and ctpD are specifically required for the optimal *in vivo* growth or survival of *M. tuberculosis* in the mouse model of tuberculosis (24). Microarray analyses of immune response induced genes have shown that ctpC and ctpV are expressed more robustly during infection of BALB/C mice, than in immune-defective SCID mice strain or during *in vitro* growth (25). Similarly, induction of ctpC, ctpG and ctpV in *M. tuberculosis* during macrophage infection has been reported (26). Linking CtpC activity with metal homeostasis during infection, early studies on *M. tuberculosis* Fe$^{2+}$-regulated genes showed that ctpC expression was induced in low-Fe$^{2+}$ media (27).

Recently, Botella et al. showed that Zn$^{2+}$ exposure induced the expression of ctpC and a ctpC-null strain was hypersensitive to Zn$^{2+}$ *in vitro* (11). *A priori*, these phenotypic characteristics might suggest that CtpC is a Zn$^{2+}$-ATPase. However, CtpC is structurally distinct from Zn$^{2+}$ transporting ATPases (20), since some of the invariant Zn$^{2+}$ coordinating residues involved in transmembrane translocation are absent from this protein. In addition CtpC also lacks characteristic, although nonessential, regulatory cytoplasmic metal binding domains. These observations suggested that further characterization was necessary to define the biochemical function of this protein. We reexamined CtpC catalytic activity and role in bacterial virulence to identify the substrate and consequent physiological role of this ATPase. Our results indicate that CtpC is a
high-affinity, slow turnover Mn\(^{2+}\)-ATPase, which is required for \textit{M. tuberculosis} virulence. CtpC appears involved in the loading of Mn\(^{2+}\) into secreted metalloproteins, such as SodA. We propose that this function may be required for infection.

**EXPERIMENTAL PROCEDURES**

**Bioinformatics analysis - \textit{M. tuberculosis}**

CtpC protein sequence was used for a BLAST search against all predicted sequence in the NCBI database. Among the returned hits, we selected those protein sequences lacking cytoplasmic metal binding domains (N- or C-MBD) and containing conserved transmembrane signatures, TM6 (CPC) and TM8 (HXXSS), likely involved in metal coordination during transport (20). Resulting homologous sequences were aligned with MUSCLE (28), Bioedit (29) and ESPript software (30). NCBI GI accession numbers were obtained and the KEGG database nomenclature was used. The proteins that were not on KEGG were named with a three-letter key to denote the organismal origin as indicated in Fig. 1.

**Bacterial Strains - \textit{M. tuberculosis} H37Rv and \textit{M. smegmatis} mc\(^2\)155**

Cells were used in these studies. These were grown at 37°C in 7H9 and 7H10 media (Becton Dickinson) supplemented with 10% OADC enrichment, or Sauton’s media as indicated in the figures. \textit{E. coli} BL21 (DE3) pLysS cells transformed with the plasmid pSJS1240 coding for rare tRNA (31) were grown at 37°C in 2XYT medium containing 50 µg/ml spectinomycin, 34 µg/ml chloramphenicol. Protein expression was induced with 0.002% L-arabinose. Cells were harvested 4 h after induction, washed with 25 mM Tris, pH 7.0, and 100 mM KCl and stored at -80°C. In alternative experiments, pBAD TOPO vector carrying \textit{M. tuberculosis} ctpC was introduced in \textit{E. coli} W3110 AzntA cells (32) and selected with 100 µg/ml ampicillin, 50 µg/ml kanamycin.

**Protein Purification - CtpC purification**

was performed according to Mandal \textit{et al.} (33). All purification steps were carried out at 0–4°C. Cells were suspended in buffer A (25 mM Tris, pH 7.0; 100 mM sucrose; 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and disrupted with a French press at 20,000 psi. Lysed cells were centrifuged at 8,000 x g for 30 min. The supernatant was then centrifuged at 229,000 x g for 1 h and the pellet membranes were resuspended in buffer A (10–15 mg/ml). For protein solubilization and purification, membranes were diluted to a final concentration of 3 mg/ml in buffer B (25 mM Tris, pH 8.0; 100 mM sucrose; 500 mM NaCl; 1 mM PMSF) and solubilized with 0.75% dodecyl-β-D-maltoside (DDM, Calbiochem). The preparation was incubated for 4 h at 4°C with mild agitation and centrifuged at 229,000 x g for 1 h. The supernatant was centrifuged overnight at 4°C with Ni\(^{2+}\)-nitrilotriacetic acid resin (Qiagen) pre-equilibrated with buffer B, 0.05% DDM, and 5 mM imidazole. The resin was washed with buffer B, 0.05% DDM containing 10 and 20 mM imidazole and the protein was eluted with buffer B, 0.05% DDM, 250 mM imidazole. Fractions were pooled, concentrated, and buffer replaced by 25 mM Tris, pH 8.0; 100 mM sucrose; 50 mM NaCl and 0.01% DDM (buffer C) using 50 kDa cut-off centricons (Millipore). The proteins were aliquoted and stored in 20% v/v glycerol at -20°C until use. All protein determinations were performed according to Bradford method (34). Purified CtpC protein was analyzed by 10% SDS-PAGE followed by Coomasie brilliant blue (CBB) staining or western blot using an anti-(His)\(_6\)-tag antibody (GenScript).
**ATPase Assays** - The ATPase activity assay mixture contained 50 mM Tris, pH 6.8, 3 mM MgCl₂, 3 mM ATP, 0.01% asolectin, 0.01% DDM, 200 mM NaCl, 0.005 mg/ml purified CtpC and either MnCl₂, CuSO₄, ZnSO₄, CdCl₂, FeCl₃, NiCl₂ or CoCl₂ as indicated in Fig. 2. ATPase activity was measured for 20 min at 37°C. Released Pi was measured (35). Curves of ATPase activity versus Ag⁺, Cu⁺, or ATP, as well as enzyme phosphorylation curves, were fit to the Michaelis-Menten equation: 
\[
v = \frac{V_{\max} L}{(L + K_{1/2})}
\]
where L is the concentration of variable ligand. The reported standard errors for \( V_{\max} \) and \( K_{1/2} \) are asymptotic standard errors reported by the fitting software KaleidaGraph (Synergy).

**Recombinneering, Mutant and Complemented Strains Preparation** - The *M. smegmatis* ctpC mutant strain was constructed following the procedures previously described (36,37). For mutation of *M. tuberculosis* ctpC, a 1000 bp fragment corresponding to the 5' - first 500 bp and 3' - last 500 bp of ctpC was constructed. An insertion cassette containing the restriction sites for NotI-HpaI-AscI was added between the 5' - and 3' - 500 bp regions. The resulting synthesized fragment was then inserted between HindIII sites in a pUC57 expression vector (GeneScript), resulting in plasmid pEL1a. Vector pKM342 contains hygR cassette flanked by NotI-AscI sites. To insert the hygR cassette in pEL1a, both pEL1a and pKM342 were digested with NotI-AscI. The 1.2 kbp hygromycin fragment was then ligated into pEL1a resulting in pEL2a. To generate ctpC mutant the resulting 2.2 kbp ctpC-hygR-ctpC fragment from digestion off pEL2a with HindIII and transformed into *M. tuberculosis* H37Rv recombinant strain. Briefly, the *M. tuberculosis* H37Rv recombinant strain bearing plasmid pNIT:ET (38) was induced for 18 h with 1 μM isovaleronitrile. The culture was treated with 0.2 M glycine for 8 hours before making electrocompetent cells and transformed. After selection on 7H10 plates containing hygromycin 50 μg/ml the presence of ctpC insertional mutation was performed by PCR amplification of the hygR cassette flanked by the N- and C-terminal junctions. All primer used are listed in Table 1.

Constructs for mutant complementation assays were made by amplifying the *M. tuberculosis* and *M. smegmatis* ctpC from genomic DNA. The resulting PCR fragments were digested and ligated into pJEBO42 (39) resulting in pJEBO42-tbC and pJEBO42-smC. The ligation reactions were transformed into DH5α cells and the presence of the insert was verified by colony PCR and restriction digests. The plasmids were then purified and transformed into the mutant strains. Transformants showing kanR were analyzed for the presence of the genes by PCR.

**Metal and Redox Stressors Sensitivity Assays** - *M. tuberculosis* H37Rv wild type, ctpC::hyg and complemented strains were grown in 7H9-OADC medium to OD_{600}=1.0. Metal sensitivity was assayed by spotting dilution series of wild type, ctpC::hyg and complemented strains on 7H10-OADC agar containing increasing concentrations of CuCl₂, CoCl₂, MnCl₂, ZnSO₄ or CdCl₂. For tert-butyl hydroperoxide (TBHP) sensitivity, 7H10-OAD was used instead of 7H10-OADC. CFU was assessed after 18 days of incubation at 37°C. *M. smegmatis* mc²155 wild type, ctpC::hyg and complemented strains were grown in 7H9-OADC medium with increasing concentrations of ZnSO₄ or MnCl₂. OD_{600} was determined after 48 h of culture. For analyzing the sensitivity to extracellular redox stress, these strains were grown in 7H9 media until OD_{600}=1.0. The cells were incubated for 0 (before addition), 30, 60, 90 and 120 min with 0.1 unit/ml xanthine oxidase and 250 μM hypoxanthine (Sigma) diluted 1:100 in phosphate buffer saline as described previously (40). Serial dilutions were plated at different time points and CFUs were quantified. Percentage of survival (CFUs at 30-120 min/CFUs at 0 min) was calculated for three independent experiments.

**Gene expression analysis** - *M. tuberculosis* H37Rv and *M. smegmatis* mc²155 cells in exponential phase were supplemented with 100 μM of various metals and redox stressors as indicated in Fig. 4 and 5 and incubated for 2 h. Chelexed Sauton’s media was used to evaluate ctpC expression under metal starvation conditions. Sauton’s media pH 7.4 containing 6 % v/v glycerol, 3.6 mM KH₂PO₄, 11.4 mM citric acid, 30 mM asparagine, 0.1 μM FeCl₃, 4.2 mM MgCl₂ and 0.05 % v/v Tween-80 was incubated over night at 4°C in constant stirring, in the presence (chelexed media) or the absence (non-chelexed media) of 1 g Chelex beads (Sigma) per 100 ml of medium. Subsequently, 0.1 μM FeCl₃ and 4.2 mM MgCl₂ were added to the media. Manganese content was determined in 7H9, non-chelexed and chelexed...
Metal Content Analysis – Whole cell metal contents were measured in *M. tuberculosis* strains grown to the late exponential phase and incubated in the presence or absence of 100 μM ZnSO₄ for 1 h. Metals bound to *M. tuberculosis* secreted proteins were measured in strains grown in Sauton’s media for 2 weeks at 37 °C. Cells were pelleted, the supernatant filtered through 0.2 μm filters (Corning) and concentrated using 3 KDa cut-off centricons (Millipore). Protein content was determined and samples prepared for metal content analysis. The corresponding bands from parallel gels showing superoxide dismutase activity were excised, de-stained by several dehydration-rehydration steps, and trypsin-digested for subsequent MS/MS analysis. Briefly, samples were dehydrated with acetonitrile and rehydrated in 100 mM NH₄HCO₃ 50 % v/v acetonitrile. When most of the dye was removed, the samples were reduced with 10 mM DTT in 100 mM NH₄HCO₃, incubated for 35 min
at 56°C in a shaking incubator and vacuum dried. Alkylation was conducted by incubating for 30 min with 55 mM iodoacetamide in 100 mM NH₄HCO₃. After wash using 100 mM NH₄HCO₃, 50 µl of 100 mM NH₄HCO₃, 50 % v/v acetonitrile were added to the pellet before final drying step. The sample was digested by adding 30-50 µl of sequencing grade Modified Trypsin (Promega) and incubated overnight at 37°C. Soluble peptides were extracted and combined with the result of three washes of 50 mL of 50% v/v acetonitrile, 5% v/v formic acid (15 min). Finally digested samples were resolved in an Accurate-Mass Q-TOF LC/MS 6520, and peptide identification from collision-induced fragmentation patterns was performed with Spectrum Mill software (Agilent).

Mice Infection - C57BL/6 female mice (8-10 weeks old) were infected with approximately 1 x 10⁷ Colony-Forming Units (CFU) of wild type, ctpC::hyg or ctpC complemented M. tuberculosis via the aerosol route. Groups of three mice were sacrificed at indicated time points and the bacterial burden in the lung homogenates were obtained by plating on 7H10 agar medium. In alternative competition experiments, two M. tuberculosis H37Rv strains (wild type and ctpC::hyg mutant or complemented) were mixed in a 3:1 ratio (6 x 10⁷ CFU in 200 µl final volume) and inoculated into the tail vein of female C57BL/6J mice. Groups of three mice were sacrificed at indicated time points and the bacterial burden in the lung homogenates, were obtained by plating on 7H10 agar medium with or without 100 µg/ml hygromycin for mutant CFU and total CFU counting, respectively. Mice were housed under specific pathogen-free conditions and in accordance with the University of Massachusetts Medical School, IACUC guidelines.

RESULTS

CtpC is a Novel ATPase - CtpC belongs to the family of P₁β-ATPases. As such, it is characterized by the presence of eight TMs in a typical membrane topology, the cytoplasmic actuator, ATP binding, and phosphorylation domains (Fig. 1A) (19,20). This last cytosolic loop contains the DKTGT sequence common to all P-type ATPases where the conserved Asp is phosphorylated during catalysis. Considering the conservation of major catalytic domains and transmembrane overall structure, it is not surprising that primary sequence of CtpC is similar (26-36% identity) to Cu⁺-ATPases and Zn²⁺ATPases, leading to hypothesis suggesting Cu⁺ or Zn²⁺ as likely substrates (11,20). BLAST searches revealed the presence of proteins with structural characteristics similar to CtpC only in a few mycobacterial species, Rhodococcus opacus, Kineococcus radiotolerans, and Nakamuraella multipartite (Fig. 1B). CtpC homologs lack the well-described regulatory cytoplasmic N-MBDs that are ubiquitous in Cu⁺- and Zn²⁺-ATPases (20). More important, CtpC proteins present differences in metal coordinating transmembrane residues. Invariant amino acids in TMs flanking the ATP binding domain are critical for the metal specificity of P₁β-ATPases, since these coordinate the metal substrates during transport (19-23). Alignment of CtpC sequences corresponding to the sixth, seventh and eighth TM segments, showed the presence of two conserved Cys in TM6, Asn and Tyr in TM7, and His, Asn and two Ser in TM8 (Fig. 1A-B). Comparison with ligand donor amino acids in substrate binding sites of Cu⁺- , Co²⁺, and particularly Zn²⁺-ATPases shows significant differences suggesting that CtpC might be involved in the transport of an alternative metal (Fig. 1C) (19-23).

CtpC is a Unique Mn²⁺-ATPase – To avoid the complexity of in vivo systems, the substrates of M. tuberculosis CtpC were biochemically characterized using purified protein. M. tuberculosis ctpC was cloned and expressed in E. coli. The resulting protein was solubilized, purified by metal affinity chromatography, and the engineered (His)₆-tag was removed by TEV-protease treatment (Fig. 2A). A functional preparation was obtained by reconstituting the protein in lipid/detergent micelles. The central characteristic of the transport mechanism of all P-ATPases is the coupling of transmembrane substrate transport to ATP hydrolysis (3,19). Stimulation of ATP hydrolysis by substrates is a proven approach to evaluate the substrate specificity of these enzymes. M. tuberculosis CtpC ATPase activity was stimulated by the presence of various metals in the assay media (Fig. 2B). Under metal saturating conditions, CtpC exhibited a maximum ATPase activity in the presence of Mn²⁺ and to a smaller extent (25-30%) by Co²⁺, Cu⁺, and Zn²⁺. It is equally significant that other metals such as Cu⁺, Ni²⁺, Fe²⁺ and Cd²⁺ did not
activate CtpC (Fig. 2B) since Zn\(^{2+}\)-ATPases are also activated by Cd\(^{2+}\) (44,45) and a role in Fe\(^{3+}\) transport might be suggested by certain phenotypic observations (see below). Cleavage of the (His)\(_{6}\) tag or the presence of thiols in the assay media had no effect on metal activation patterns (data not shown). Fig. 2C shows the dependence of CtpC ATPase activity on the Mn\(^{2+}\) and Zn\(^{2+}\) concentration. Interestingly, CtpC turnover (V\(_{\text{max}}\)) (Table 2) is remarkably slow when compared to enzymes involved in metal detoxification and tolerance such as E. coli CopA (10-fold higher V\(_{\text{max}}\)) (46), P. aeruginosa CopA1 (4-fold higher) (41), E. coli ZntA (10-fold higher) (44), or A. fulgidus CopA (4-fold higher) (33). In fact, CtpC V\(_{\text{max}}\) is more similar to P. aeruginosa CopA2, a slow transporting Cu\(^{2+}\)-ATPase involved in cytochrome c oxidase assembly (41). The apparent high affinity of CtpC for Mn\(^{2+}\) is also consistent with this function. Although the estimation of relative metal affinities requires equilibrium binding experiments and the low ATPase activity hampers precise activation K\(_{1/2}\) determinations, it is clear that CtpC interacts with Mn\(^{2+}\) and Zn\(^{2+}\) with very high affinities (Table 2). CtpC estimated K\(_{1/2}\) for activation is approximately 1-2 orders of magnitude lower than those seen in other P. \(\text{IB}^{-}\)-ATPases (33,44,46,47). These observations were further confirmed by analysis of the homologous M. smegmatis CtpC. The heterologously expressed and purified M. smegmatis protein showed metal activation and biochemical parameters identical to those of the M. tuberculosis CtpC (Table 2).

Invariant amino acids located in TMs determine metal specificity and, as metal ligands, are required for enzyme function (19-23). While a full analysis of metal coordination by CtpC is beyond this report, the importance of the distinct HXXSS sequence in TM8 of M. tuberculosis CtpC was explored. As expected, proteins carrying point H699A and neighboring S700A/S701A showed no ATPase activity at saturating Mn\(^{2+}\) levels (Fig. 2D). This supports the link between the observed Mn\(^{2+}\) dependent ATPase activity and the binding of this metal to a transmembrane transport site distinct from those in Cu\(^{2+}\) - or Zn\(^{2+}\)-ATPases.

The observed biochemical properties, low transport rate and high apparent metal affinity, suggest that CtpC might perform a role other than detoxifying the cells by exporting cytoplasmic metals into extracellular media (3,41). However, the modest activation CtpC by Zn\(^{2+}\) and the previously proposed role for this protein as a Zn\(^{2+}\)-ATPases controlling cytoplasmic levels of this metal (11), prompted us to confirm that this protein’s specificity was not altered in vivo. To test this, we complemented a zntA::kan RW3110 E. coli strain, lacking the functional Zn\(^{2+}\)-ATPase (32), with M. tuberculosis CtpC. Fig. 3A shows that, while Zn\(^{2+}\) tolerance is restored by complementing E. coli with ZntA, M. tuberculosis CtpC introduced in a similar construct, was unable to rescue the E. coli zntA mutant when grown in the presence of high Zn\(^{2+}\) levels. Fig. 3B shows the expression of CtpC under the tested conditions. These results indicate that CtpC is unlikely to play a role in controlling cytoplasmic Zn\(^{2+}\) levels.

CtpC deficiency renders M. tuberculosis sensitive to Zn\(^{2+}\) and oxidative stress - It has been reported that deletion of ctpC decreases the Zn\(^{2+}\) tolerance of M. tuberculosis GC1257 strain (11). We observed a similar phenotype in M. tuberculosis H37Rv ctpC::hyg cells when grown at Zn\(^{2+}\) concentrations as low as 5 µM (Fig. 4A). However, no changes were observed in the sensitivity to Cd\(^{2+}\), a metal that usually shows similar toxicity in Zn\(^{2+}\) mutants, or to the enzyme substrate Mn\(^{2+}\) (Fig. 4B and 4C). Similarly, the presence of Co\(^{2+}\) or Cu\(^{2+}\) in the media had no effect on the growth of these cells (data not shown). Reasoning that Zn\(^{2+}\) could impose oxidative stress, we determined if the ctpC mutant was sensitive to other oxidants. Indeed, we found that this mutant was hypersensitive to both tert-butyl hydroperoxide (TBHP; Fig. 4D) and superoxide (generated with hypoxanthine/Xanthine oxidase; Fig. 4E). In both cases, the wild type phenotype was restored in the complemented strain. Correlating with the alterations in Zn\(^{2+}\) tolerance, a significant induction of ctpC expression in response Zn\(^{2+}\) was detected (Fig. 4F) (11). This induction was not observed when cells were challenged with other tested stressors (Fig. 4F). These data implies a complex system in which ctpC expression is not induced by, nor confers resistance to, its substrate Mn\(^{2+}\). However, the enzyme is required for tolerance to Zn\(^{2+}\) and oxidative stress. To explain these observations, the role of CtpC in M. smegmatis was studied. Surprisingly, the M. smegmatis ctpC::hyg strain showed no higher sensitivity to Zn\(^{2+}\) (Fig. 5A), although it behaved...
as the M. tuberculosis mutant in the presence of Mn$^{2+}$ (no effect; Fig. 5B) and redox stress (increased sensitivity; Fig 5C). Furthermore, contrary to the observation that Zn$^{2+}$ stimulates M. tuberculosis ctpC expression, no induction was observed in M. smegmatis when exposed to the metal (Fig. 5D). Interestingly, a 15-fold increase in ctpC expression was observed when M. smegmatis was exposed to the extracellular superoxide generator hypoxanthine/xanthine oxidase.

*Deletion of ctpC leads to cytoplasmic Mn$^{2+}$ accumulation and a decrease in secreted Mn$^{2+}$ bound proteins* – Based on the role of well described P$_{1B}$-ATPases in controlling cellular metal quotas, the content of various transition metals in ctpC mutant M. tuberculosis cells was measured (Table 3). Under basal conditions, no changes in transition metal levels were observed except for a modest but significant increase of Mn$^{2+}$ content. Considering the described Zn$^{2+}$ accumulation in M. tuberculosis CG1237 ctpC mutant cells (11), Zn$^{2+}$ levels under similar Zn$^{2+}$ stress conditions were also analyzed. No Zn$^{2+}$ accumulation was detected in our system (Table 3, 5). Mn$^{2+}$ homeostasis was further analyzed in cells growth in the presence of 50 μM Mn$^{2+}$. Taking into account the proposed function of P$_{1B}$-ATPases on the metallation of periplasmic/secreted proteins, Mn$^{2+}$ bound to periplasmic/secreted proteins fractions was measured. To avoid interference from the presence of albumin in 7H9 medium, these experiments were performed in the more defined Sauton’s medium. A four-fold increase in cellular Mn$^{2+}$ content was observed in the M. tuberculosis ctpC mutant, along with a significant decrease in the Mn$^{2+}$ bound to secreted proteins (Fig. 6A, Table 4). On the contrary, the levels of Zn$^{2+}$, Fe$^{2+}$ or Cu$^{2+}$ bound to secreted proteins, were not affected in the M. tuberculosis or M. smegmatis ctpC mutant strain (Table 4, 5). M. smegmatis ctpC mutant cells allowed further analysis of subcellular fractions (Fig. 6B). A large increase of Mn$^{2+}$ bound to cytosolic proteins was observed, along with a significant decrease in Mn$^{2+}$ bound to secreted proteins. These results confirmed the function of CtpC as a Mn$^{2+}$-ATPase involved not only in maintaining cytoplasmic metal quotas but likely participating in the metallation of secreted Mn$^{2+}$-proteins. Moreover, these observations suggest that CtpC might not be involved in conferring Zn$^{2+}$ tolerance but rather in an alternative process, such as the response to redox stress. In the case of M. tuberculosis, this response to redox stress might be triggered by high Zn$^{2+}$ levels.

*CtpC is required for functional secreted SodA* – The metallation of secreted Mn$^{2+}$-bound proteins appeared to be deficient in ctpC mutants and some of these may be required for overcoming redox stress. Mn-SodA was a logical candidate for CtpC-mediated metallation. In mycobacteria, the SodA protein is secreted in the apo-form via the Sec pathway (18). Consequently, SodA probably acquires its metal in the extracellular milieu. The metal specificity of SodA-like enzymes is not predictable based on primary protein sequence. While M. tuberculosis SodA appears to contain Fe$^{2+}$ other highly homologous SodA’s are Mn$^{2+}$ enzymes (17,48,49). Moreover, cambialistic properties have been observed in these enzymes, suggesting that distinct metals might be used by the same enzyme under different conditions. To test the putative role of CtpC in the metallation of secreted SodA, in-gel superoxide dismutase activity assays were performed. The secreted fraction from M. tuberculosis ctpC::hyg strain showed a 37% decrease in the activity of SodA compared to wild type (Fig. 7A, B). The SodA activity was partially recovered in the CtpC complement strain when compared to wild type, probably reflecting the levels of CtpC expression in the complemented cells. Interestingly, when analyzing the secreted SodA from M. smegmatis ctpC::hyg mutant, the activity decreased up to 80% compared to the SodA obtained from the wild type strain (Fig. 7C, D). CB staining of identical gels showed no decrease SodA protein secretion (Fig. 7C, lower panel). The identity of secreted SodA was verified by liquid chromatography/mass spectrometry (LC/MS; Table 6). Since mycobacteria also have cytoplasmic Cu/Zn-SodCs, it is also relevant that no differences were found when analyzing total superoxide dismutase activity in whole cell homogenates, cytosolic, or membrane fractions obtained from M. smegmatis wild type, ctpC::hyg and the complemented strains (data not shown).

Because of the mentioned cambialistic properties of SodA (50-52), we determined if M. smegmatis SodA activity could be restored by other metal co-factors independently of CtpC
activity. *M. smegmatis* wild type, *ctpC::hyg* and the complemented strains were grown in Sauton’s media supplemented with 50 μM MnCl₂, FeCl₂, ZnSO₄ or CoCl₂ until late log phase. In-gel Sod activity assays showed that SodA activity was restored when adding MnCl₂ or FeCl₂ to the culture media of *M. smegmatis ctpC::hyg* mutant strain (Fig. 7C). The addition of ZnSO₄ and CoCl₂ had no effect on this activity. No differences were observed in the activity of the secreted SodA from the wild type and complemented strains (data not shown). These data further supports an important role for CtpC in the Mn²⁺ metallation of mycobacterial SodA during metal starvation stress. Fe²⁺ metallation appears to be unaffected, and under certain culture conditions this prosthetic group may be used (17). The alternative use of these metal cofactors by SodA is addressed the discussion.

Considering the requirement of CtpC for Mn²⁺ secretion and SodA metallation, and the lack of *ctpC* induction under Mn²⁺ excess (Fig. 4F and 5D), it is tempting to hypothesize that under Mn²⁺ starvation *ctpC* expression should be induced. Although all the media used in these studies have no Mn²⁺ formally included; interestingly, a 3.0 ± 0.3 and a 2.12 ± 0.06 fold increase in *ctpC* expression was observed, when *M. smegmatis* cells were cultured in chelexed (0.3 nM Mn²⁺) or non-chelexed (0.4 nM Mn²⁺) Sauton’s media respectively, compared to those maintained in 7H9 media (0.9 nM Mn²⁺). While small changes in Mn²⁺ levels might affect *ctpC* transcription, these are very small and unlikely to be of physiological significance.

**CtpC is important for *M. tuberculosis* virulence** - Large-scale genetic studies predicted that *ctpC* is required for growth or survival of *M. tuberculosis* H37Rv in the C57BL/6 mouse model of tuberculosis (24). More recent studies reported that mutation of the *ctpC* gene impairs the intracellular growth *M. tuberculosis* strain GC1237 in human macrophages, but does not alter the virulence of this strain in BALB/C or SCID mice (11). To verify the importance of CtpC for *M. tuberculosis* virulence, we assessed the growth of the *ctpC::hyg* mutant strain in two different models of tuberculosis. First, *M. tuberculosis* H37Rv, *ctpC::hyg*, and *ctpC* complemented strains were used to infect the relatively resistant C57BL/6 mouse strain via the low dose aerosol route. The *ctpC::hyg* strain grew similarly to wild type *M. tuberculosis* during the initial phase of infection that is governed by innate immune mechanisms. However, a significant decrease in viable *ctpC::hyg* bacteria was noted after a month of infection (Fig. 8A). This specific persistence defect is similar to mutants that are unable to resist the adaptive immune response (53). The attenuation of this mutant was confirmed using a more sensitive competitive model in which the ratio of mutant versus wild type bacteria was monitored in animals that were co-infected via the intravenous route. As we observed in the single-strain infections, the *ctpC::hyg* mutant was significantly underrepresented after 42 days of infection (Fig. 8B). The attenuation of this strain was observed at earlier time points in the competitive model (21 days), likely due to the earlier onset of adaptive immunity after intravenous challenge (54). No differences in growth were observed between wild type and complemented strains in vitro (not shown), and genetic complementation reversed the *in vivo* growth/survival defects of this mutant. Thus, *ctpC* is required for the bacterium to adapt to the host environment, and appears to be specifically important for resisting the adaptive immune response.

**DISCUSSION**

Large-scale genetic screens implicated *ctpC* in the adaptation of *M. tuberculosis* to the host environment (24). Recent studies have shown that an *M. tuberculosis ctpC* mutant is sensitive to Zn²⁺ (11), a metal that appears to accumulate in the phagosome (9). Taking into account these phenotypes and CtpC homology with Cu⁺- and Zn²⁺-ATPases, it was proposed that CtpC was a Zn²⁺-ATPase involved in the efflux of cytoplasmic Zn²⁺ (11). However, CtpC lacks signature residues part of the TM-MBS of Cu⁺- and Zn²⁺-ATPases suggesting that it might transport alternative metals. Considering its putative importance for virulence and unique structure, the function of CtpC in *M. tuberculosis* and *M. smegmatis* was examined using a combination of biochemical and genetic approaches. Here, we describe that CtpC shows a preference for Mn²⁺, controls the Mn²⁺ cytoplasmic quota, and is involved in the uploading of Mn²⁺ into secreted metalloproteins.
We propose that these activities of CtpC are important for *M. tuberculosis* virulence.

CtpC is a metal transport P_{1B}-ATPase with characteristic phosphorylation domains and membrane topology, although it lacks the regulatory N-MBDs ubiquitous in enzymes with specificity for Cu^{+} or Zn^{2+} (19,20). It is now well established that metal specificity is conferred by conserved residues in the TMs flanking the large cytoplasmic ATP binding and hydrolysis domain (19-23). Consequently, in spite of the significant similarity between CtpC and Cu^{+}/Zn^{2+}-ATPases, it lacks the sets of invariant metal coordinating residues found in well-characterized P_{1B}-ATPases, particularly the Lys, Asp in TM7 and TM8 that characterize Zn^{2+}-ATPases (20,22). The analysis of the thirteen sequences of homologous CtpC proteins showed that while these have retained the CPC signature in TM4, they have unique NY in TM7 and HNASS in TM8. The presence of these unique residues is not trivial since their replacement leads to inactive proteins. It is interesting that while these residues resemble the Cu^{+} binding sequences (19-21), the replacement of Met for His (a harder Lewis base) is consistent with selectivity for Mn^{2+} (a hard Lewis acid), the substrate of CtpC identified in our studies. Moreover, the Ser (a hard Lewis base) conserved in CtpC provides further specificity towards Mn^{2+}. This Ser is absent in Zn^{2+} sites (55,56).

Direct biochemical analysis of CtpC activity showed that maximum activation of the transporter occurs in the presence of Mn^{2+}. However, Zn^{2+}, Co^{2+}, and even Cu^{2+}, can act as alternative substrates although at slower turnover rates. This is not surprising, since many Zn^{2+}-ATPases and Cu^{+}-ATPases can transport alternative metals sharing similar chemical properties (33,44,47,57). Furthermore, it is significant that CtpC is not activated by Cd^{2+}, a well-characterized activator of Zn^{2+}-ATPases (44,58). However, it is perhaps more important to consider the high apparent affinity of CtpC for Mn^{2+} and that its activity is relatively slow compared to that of enzymes responsible for maintaining cytoplasmic metal quotas. If the reported activity of *E. coli* Zn^{2+}-ATPase is considered, the Zn^{2+}-ATPase activity of CtpC is two orders of magnitude lower when measured under practically identical conditions. This kinetic difference likely explains the inability of CtpC to confer Zn^{2+} tolerance and functionally complement a ΔzntA *E. coli* mutant. We hypothesize that in *M. tuberculosis* the necessary Zn^{2+} efflux is mediated by the cation diffusion facilitator (CDF) coded by Rv2025. This is homologous to the *M. smegmatis* Zn^{2+} transporter ZitA (59) and *E. coli* ZitB (60).

While biochemical determinations provide a direct evaluation of heavy metal transport ATPase substrates, characterization of cellular roles might provide further insight into the enzyme selectivity. Determination of metal contents and tolerance to metal stress, as well as induction of gene expression by putative substrates are commonly used indicators of substrate specificity. We observed little or no sensitivity of the ctpC::hyg cells to various metal stressors, except for the described lack of tolerance to Zn^{2+} (11). This phenotype was not observed in *M. smegmatis*, suggesting that *M. tuberculosis* is particularly sensitive to Zn^{2+} or to an associated stress (see below). Interestingly, the lack of Zn^{2+} tolerance was correlated by lack of induction of the ctpC gene by Zn^{2+} in *M. tuberculosis*. On the other hand, it was also remarkable that the Cd^{2+} did not induce ctpC expression, suggesting that ctpC expression is not driven by Zn^{2+} sensing transcriptional regulators (61).

We observed that mutation of ctpC led to an increase in intracellular Mn^{2+} levels, but did not affect the cellular Zn^{2+}, Co^{2+} or Fe^{2+} content. Mn^{2+} is apparently nontoxic to the cells, as high (millimolar) extracellular Mn^{2+} is necessary to affect cell grow. Noticeably, the Mn^{2+} accumulation was observed even in basal media conditions, it was more prominent in cells grown in 50 µM Mn^{2+}. These findings are consistent with our biochemical data, although we did not observe the previously described Zn^{2+} accumulation in ctpC mutant cells under Zn^{2+} stress conditions (11). These differences might be associated with alternative Zn^{2+} efflux systems present in the different strains of *M. tuberculosis* used in these experiments. Previous work employed the *M. tuberculosis* GC1237 strain, while we characterized the ctpC::hyg mutant in the H37Rv background. Still, our *in vivo* observations support the conclusion that this CtpC functions primarily as a Mn^{2+} transporter.

Given this biochemical function, the Zn^{2+} sensitivity and the Zn^{2+}-dependent ctpC expression observed in *M. tuberculosis* are unexpected. It
might be considered that metal homeostasis is linked to other cellular phenomena, for instance the redox equilibrium. In particular, the association of Zn$^{2+}$, Mn$^{2+}$, and Fe$^{2+}$ transporters in several redox responsive regulons has been documented (62,63). Expression of the ctpC gene is induced in low Fe$^{3+}$ conditions (27) and it is possible that high Zn$^{2+}$ concentrations might mimic Fe$^{2+}$ deficiency. Alternatively, a link between Zn$^{2+}$ and σ factors has been proposed in M. tuberculosis (64), and ROS have been shown to affect the signaling pathways involved in the activation of multiple transcription factors (65). Considering the observation that ctpC:::hyg cells are also sensitive to redox stressors, it is tempting to speculate that the sensitivity to Zn$^{2+}$ is related to alteration in Fe$^{3+}$ or redox homeostasis rather than to an increase in cytosolic Zn$^{2+}$. The increased susceptibility of both mycobacterial ctpC::hyg mutants to the oxidative stress produced by xanthine oxidase support these ideas.

An alternative link between Zn$^{2+}$ and redox stress might also be considered. In M. tuberculosis metallothioneins are primary barriers against Cu$^{+}$ toxicity (66). There is evidence that these proteins also bind Zn$^{2+}$ and Cd$^{2+}$ with high affinity. Both ions could generate an imbalance in cell redox throughout mechanisms such as mycothiol consumption, thioredoxins inhibition, or Cu$^{+}$ displacement by competition when present at high concentrations (67). Addition of Zn$^{2+}$ to the media might release Cu$^{+}$ from oxidized metallothioneins, the former generating ROS by means of the Fenton reaction. Contrary to Zn$^{2+}$, 50 μM Cd$^{2+}$ induces the transcription of metallothioneins in M. tuberculosis (66). The induction of metallothioneins by Cd$^{2+}$ might explain why M. tuberculosis ctpC is not sensitive to Cd$^{2+}$ when compared to the wild type strain H37Rv.

CtpC is required for M. tuberculosis growth and survival in the mouse model. The importance of Mn$^{2+}$ for the virulence of other bacterial pathogens has been described, although Mn$^{2+}$ levels in the phagosome might not change dramatically during infection (9,49). Our biochemical and phenotypic analyses indicate that CtpC, although partially influences cytoplasmic Mn$^{2+}$ levels, it is an slow ATPase, its expression is not induced by its substrate, and CtpC-deficient cells exhibit normal tolerance to Mn$^{2+}$ metal excess. These are all characteristics similar to those observed in Cu$^{+}$-ATPases responsible for assembly of cytochrome c oxidase, instead of cytosolic detoxification (41). In agreement with this putative role, the ctpC:::hyg mutant showed a decrease in the amount Mn$^{2+}$-bound to secreted proteins. Further experiments, showed that deletion of ctpC reduced secreted Mn$^{2+}$/Fe$^{2+}$ SodA activity without altering the abundance of the protein, suggesting that the enzyme might not be metallated. Supporting this hypothesis, M. smegmatis SodA activity was restored by supplementing the growth media with either Mn$^{2+}$ or Fe$^{2+}$. Secreted metalloproteins such as SodA have been shown to be required for virulence (14,16). Both M. tuberculosis and M. smegmatis SodA are highly homologous, and lack a classical signal sequence for protein export (68), though M. tuberculosis SodA is exported via the SecA2 pathway which exports unfolded apo-proteins (18). Interestingly, one important difference between them is the metal co-factor either SodA acquires in vitro. M. tuberculosis SodA has been characterized as a Fe-Sod, while M. smegmatis SodA contains Mn$^{2+}$ (17). However, M. smegmatis SodA has a cambialistic behavior in vitro, being activated by Fe$^{3+}$ when the pH is acidic (48). These enzymes differ in one of the metal coordinating residues; M. tuberculosis contains a His instead of a Gln at residue 145, which is present in M. smegmatis Mn$^{2+}$ SodA. However, replacing His145 to Gln does not affect the metal binding in M. tuberculosis, as determined by x-ray crystallography (17). The specificity for iron of the tuberculosis SodA seems to be counter-intuitive, considering that upon macrophage infection, M. tuberculosis cells must face and overcome iron starvation (8). Thus, it is possible that M. tuberculosis SodA is preferentially loaded with Mn$^{2+}$ in the iron-limited in vivo environment.

In summary, the data presented indicates that CtpC is unique Mn$^{2+}$-ATPase predominantly present in mycobacteria. The enzyme appears to be required for loading of Mn$^{2+}$ secreted metalloproteins; in particular M. smegmatis, and possibly M. tuberculosis, SodA. In this role, the CtpC is a key element for virulence.

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FOOTNOTES

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4The abbreviations used are: TM, transmembrane segment; TM-MBS, transmembrane metal binding site; Ctp, cation transporter protein; Sod, superoxide dismutase; TEV, Tobacco Etch Virus; Mtb, Mycobacterium tuberculosis; Msm, Mycobacterium smegmatis; XO, xanthine oxidase; HE haematoxylin eosin staining; N- or C-MBD, cytoplasmic metal binding domains; ROS, reactive oxygen species; RNS,
reactive nitrogen species; TBPH, tert-butyl hydroperoxide; CBB, coomasie brilliant blue; DDM, dodecyl-β-D-maltoside; PMSF, phenylmethylsulfonyl fluoride.

For simplicity P-type ATPases will be referred as P-ATPases, P1B-ATPases, etc.

FIGURE LEGENDS

FIGURE 1. CtpC structure. (A) Membrane topology of \textit{M. tuberculosis} CtpC and location of the conserved residues in TM6, TM7 and TM8 of CtpC. (B) Partial alignment of CtpC homologous proteins sequences corresponding to TM6 and phosphorylation site DKTGT (upper panel) and TM7 and TM8 (lower panel). Sequences are from Rv3270, \textit{M. tuberculosis}; MSMEG\_6058, \textit{M. smegmatis} str. MC\_S155; Krad\_0290, \textit{Kineococcus radiotolerans} SRS30216; Namu\_3854, \textit{Nakamurella multipartite}; ROP\_04370, \textit{Rhodococcus opacus} B4; MLBr\_00747, \textit{M. leprae} Br4923; Mit\_254821357, \textit{M. intracellulare} ATCC 13950; Mcb\_342861474, \textit{M. colombiense} CECT 3035; MAV\_4235, \textit{M. avium} 104; Mb3298, \textit{M. bovis} AF2122/97; MCAN\_32891, \textit{M. canetti}; Mkn\_240172497, \textit{M. kansasii}; MMAR\_1271, \textit{M. marinum}. Putative TM segments (black bars) and likely metal coordinating amino acids (red rectangles) are indicated. (C) Comparison of metal coordinating residues of CtpC, Cu\(^{2+}\), Zn\(^{2+}\), and Co\(^{2+}\)-ATPases.

FIGURE 2. Biochemical characterization of \textit{M. tuberculosis} CtpC. (A) CtpC purification. 20 µg CtpC, treated and untreated with TEV protease, resolved in SDS-PAGE, and CBB or immunostained (WB). (B) CtpC ATPase activity in the presence of 10 nM metals (saturating concentration). 100% = 1.1 µmol/mg/h. Curves were fit to \(v = V_{\text{max}} L / (L+K_{i,v})\), where \(L\) is the concentration of variable ligand. Data obtained at 10 µM and 100 µM metal are not shown but were included in curve fitting. Fitting parameters are presented in Table 2. (D) Mn\(^{2+}\)-ATPase activity of H697A and S700A/S701A CtpC mutants. In all experiments, data points are the mean ± SE of at least three independent experiments performed in duplicate.

FIGURE 3. Lack of functional complementation of \(\Delta zntA\) \textit{E. coli} by ctpC. (A) Effect of Zn\(^{2+}\) on the growth of \textit{E. coli} W3310 wild type (●), \(\Delta zntA\) (○), \(\Delta zntA\) complemented with \textit{E. coli} zntA (◇) and \textit{M. tuberculosis} ctpC transformed \(\Delta zntA\) strains (■ uninduced; △ arabinose induced). (B) Expression of CtpC in transformed \(\Delta zntA\) \textit{E. coli} cells. Dot blotted cells immunostained with anti-(His)\(_6\)-tag antibody.

FIGURE 4. Response of \textit{M. tuberculosis} ctpC::hyg strain to metal and redox stressors. (A-D) \textit{M. tuberculosis} H37Rv wild type (●), ctpC::hyg (○) and complemented (■) strains were grown in the presence of increasing concentrations of the indicated stressor, plated on 7H10 agar plates, CFUs counted and normalized by ml of culture. (E) \textit{In vitro} susceptibility of \textit{M. tuberculosis} H37Rv wild type (●), ctpC::hyg (○) and complemented (■) strains to extracellular redox stress. Cells were treated with 250 mM hypoxanthine and 0.1 unit/ml xanthine oxidase and plated on 7H10 agar plates at different time points. (F) Induction of ctpC expression in \textit{M. tuberculosis} H37Rv cells incubated for 2 h with 100 µM CuSO\(_4\), ZnSO\(_4\), CoCl\(_2\), MnCl\(_2\), TBHP, FeCl\(_2\) or 1 h with 250 mM hypoxanthine and 0.1 unit/ml xanthine oxidase. Samples were processed for qPCR analysis and normalized against \(\text{sigA}\). Data are the mean ± SE of three independent experiments.

FIGURE 5. Response of \textit{M. smegmatis} ctpC::hyg strain to metal and redox stressors. \textit{M. smegmatis} wild type (●), ctpC::hyg (○) and complemented (■) strains were grown in 7H9 media supplemented with increasing concentrations of (A) Zn\(^{2+}\) or (B) Mn\(^{2+}\) for 48 h and OD\(_{600}\) was determined. (C) \textit{In vitro} susceptibility of \textit{M. smegmatis} wild type (●), ctpC::hyg (○) and complemented (■) strains to extracellular redox stress. Cells were treated with 250 mM hypoxanthine and 0.1 unit/ml xanthine oxidase and plated on 7H10 agar plates at different time points. (D) Induction of ctpC expression in \textit{M. smegmatis} wild type cells incubated for 2 h with 100 µM CuSO\(_4\), ZnSO\(_4\), CoCl\(_2\), MnCl\(_2\), TBHP, FeCl\(_2\) or 1 h with 250 mM hypoxanthine and
0.1 unit/ml xanthine oxidase. Samples were processed for qPCR analysis and normalized against sigA. Data are the mean ± SE of three independent experiments.

**FIGURE 6.** Effect of *ctpC* deletion on protein bound Mn\(^{2+}\) homeostasis *M. tuberculosis* (A) and *M. smegmatis* (B) wild type (black bars), *ctpC::hyg* (white bars), and complemented (gray bars) strains were grown in the presence of 50 µM Mn\(^{2+}\). The protein bound Mn\(^{2+}\) content in different cellular fractions was determined by furnace AAS. Significant differences from the wild type as determined by Student’s t test are indicated. * P ≤ 0.008; ** P ≤ 0.002.

**FIGURE 7.** Effect of *ctpC* deletion on the activity of secreted SodA. (A) In-gel SodA activity following separation of *M. tuberculosis* secreted protein fraction in non-denaturing PAGE (upper panel). Similar gels were CBB stained to visualize protein levels (lower panel). (B) Densitometry of *M. tuberculosis* SodA activity bands. (C) In-gel SodA activity following separation of *M. smegmatis* secreted protein fraction in non-denaturing PAGE (upper panel). Similar gels were CBB stained to visualize protein levels (lower panel). The mutant *ctpC::hyg* strain was grown in 50 µM of the indicated metals. (D) Densitometry of *M. smegmatis* SodA activity bands. Data are the mean ± SE of at least three independent experiments. Significant differences from the wild type as determined by Student’s t test are indicated. * P ≤ 0.002; ** P ≤ 0.001.

**FIGURE 8.** CtpC is required for *M. tuberculosis* virulence. (A) *M. tuberculosis* H37Rv growth fitness of wild type (●), *ctpC::hyg* (♦), and complemented (■) strains after 21 and 28 days of infection. (B) Relative *in vivo* growth rates of wild type and *ctpC::hyg* *M. tuberculosis* H37Rv strains after 21 and 42 days of mice infection. Means ± SE from three to five mice are shown. Significant differences from the wild type as determined by Student’s t test are indicated. * P < 0.015; ** P < 0.01.
Figure 1

A

A-domain

ATP-binding domain

B

CtpC a mycobacterial Mn$^{2+}$-ATPase

C

|       | TM6 | TM7 | TM8 |
|-------|-----|-----|-----|
| CtpC  | CPC | NY  | HNXSS |
| Zn$^{2+}$-ATPases | CPC | K   | D   |
| Cu$^{2+}$-ATPases | CPC | YN  | MXXSS |
| Co$^{2+}$-ATPases | SPC | HEXXT |
Figure 2
Figure 3

A

B

\( \Delta \text{zntA} \quad \Delta \text{zntA} + \text{clpC} \quad \text{wt} \quad \Delta \text{zntA} + \text{zntA} + \text{clpC} + \text{arabinose} \)
Figure 4
Figure 5

(A) OD<sub>560</sub> vs. [Zn<sup>2+</sup>] (mM)
(B) OD<sub>560</sub> vs. [Mn<sup>2+</sup>] (mM)
(C) % Survival vs. Time (min) for Hypoxantine/Xanthine oxidase
(D) Fold increase comparison for Zn<sup>2+</sup>, Mn<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>, and Co<sup>2+</sup>.
CtpC a mycobacterial Mn$^{2+}$-ATPase

Figure 6
Figure 7
Figure 8
Table 1. List of primers used in this study

| Primer name      | Sequence 5'-3'                                      | Use                                                                 |
|------------------|-----------------------------------------------------|----------------------------------------------------------------------|
| Mtb Fwd          | 5'-ACCAGTGCTCGCCGAACAGCTTGTA-3'                     | Amplification of the N-terminal regions of Mtb ctpC                 |
| ctpC::hyg out2   |                                                     |                                                                      |
| Mtb Rvs          | 5'-TGCACGGGACCAACCTTCTGGG-3'                        | Amplification of the C-terminal regions of Mtb ctpC                 |
| ctpC::hyg CatC-  |                                                     |                                                                      |
| prEL8            | 5'-TCGTAAACGACCGGAAGAAGTACAG-3'                     | Amplification of the M. tuberculosis rv3269-ctpC operon             |
| prEL9            | 5'-ATGGTACCCGAACGTGTGAATTGAC-3'                     | Amplification of the M. tuberculosis rv3269-ctpC operon             |
| Mtb ctpC Fwd     | 5'-ACCCTGGAAGTGGTATCGGACG-3'                        | Clone Mtb ctpC in pBAD                                              |
| Mtb ctpC Rvs     | 5'-GCGGCTCCAGGGGTAAGGGAT-3'                         | Clone Mtb ctpC in pBAD                                              |
| Mtb ctpC ANASS Fwd | 5'-GCGGGGTATCTGTGTAACCGTCGTCG-3'                     | Mutation of the TM-MBD (TM8) of Mtb ctpC                            |
| Mtb ctpC ANASS Rvs | 5'-CGACGACGCTTACAGGATAGCGGC-3'                     | Mutation of the TM-MBD (TM8) of Mtb ctpC                            |
| Mtb ctpC HNAAA Fwd | GCGATCTCTGCACACCGGTCTGTGGACGTGCTG-3'                 | Mutation of the TM-MBD (TM8) of Mtb ctpC                            |
| Mtb ctpC HNAAA Rvs | CACCACCCACAGCGGATGTTGCAGTCG-3'                     | Mutation of the TM-MBD (TM8) of Mtb ctpC                            |
| Mtb qFwd sigA    | 5'-CTCGGTTGCCTACCTCA-3'                             | M. tuberculosis Housekeeping gene for qPCR analysis                 |
| Mtb qRvs sigA    | 5'-GCGGCCTGTAAGCTCGTA-3'                            | M. tuberculosis Housekeeping gene for qPCR analysis                 |
| Mtb qFwd ctpC    | 5'-TCGACGCATCGTGTTCG-3'                             | M. tuberculosis ctpC gene for qPCR analysis                         |
| Mtb qRvs ctpC    | 5'-CTCGGCTCCCAAATCTTTATG-3'                         | M. tuberculosis ctpC gene for qPCR analysis                         |
| Smeg6058-F       | 5'-GTGCGCTGATGTACCGGCTAGACCGGGCGGCCCGCATGCGGTACAGGCGGCTTCTAGAAC TAGTGGAA-3' | Hyg cassette amplification with 50 bp of 5'-flanking region locus MSMEG_6058 added to the 5'-end |
| Smeg6058-R       | 5'-CTAGTGCATTTCGTACCAGGATACGCCGGGCCGCGGCGCTTGACCACATACGTGAAGCTGAGACGATCGA CTTCT-3' | Hyg cassette amplification with 50 bp of 3'-flanking region locus MSMEG_6058 added to the 3'-end |
| MSMEG_6058-F     | 5'-CATAGTTGAGGAAGTGGCGGCGGCGGCACGAGCAGCAGCAGCTGAGT GCACCCTGAGGATCTGGAACGCTG-3' | Hyg cassette amplification with 125 bp of 5'-flanking region locus MSMEG_6058 added to the 5'-end |
| **MSMEG_6058-R** | 5'-GGCGGCCGACATGGTGAGCAGCCTGGGCGGTACCGAGCGGCTGTTGCAACACCGGCGTGCTTCCGCAACGGCCTAGTGATTTCTGATCCGGGA-3' | Hyg cassette amplification with 125 bp of 3'-flanking region locus MSMEG_6058 added to the 3'-end |
|------------------|-------------------------------------------------|-------------------------------------------------|
| **intSmeg6058-For** | 5'-TTGATTTCTTTGGGATACGAGTTGGAATCCGTTGTTCGCGGGACATGGTGAACGCGCCGGTGGCGGTACCGAGCGGCTGTTGCAACACCGGCGTGCTTCCGCAACGGCCTAGTGATTTCTGATCCGGGA-3' | Deletion MSMEG_6058 verification |
| **intSmeg6058-Rev** | 5'-ATGATGGCAGCCTATGGTTGAT-3' | Deletion MSMEG_6058 verification |
| **Smeg6058-V1** | 5'-GTTGATGGCAGCCTATGGTTGAT-3' | Deletion MSMEG_6058 verification |
| **Smeg6058-V2** | 5'-GTTGATGGCAGCCTATGGTTGAT-3' | Deletion MSMEG_6058 verification |
| **Fwd EcoRI ctpC** | 5'-ACTGGGAATTCGCTGTACGTCGACGGATC-3' | Clone SMEG_6058 in pJEB402 |
| **Rvs Hpal ctpC** | 5'-ACTGGGAATTCGCTGTACGTCGACGGATC-3' | Clone SMEG_6058 in pJEB402 |
| **ctpC_smeg fwd** | 5'-CTAGTCGATTTCTGATCCGGGATCAG-3' | Clone SMEG_6058 in pBAD |
| **ctpC_smeg rvs** | 5'-CTAGTCGATTTCTGATCCGGGATCAG-3' | Clone SMEG_6058 in pBAD |
| **Msm_2758 qFwd sigA** | 5'-GAAGACACCGACCTGGAACT-3' | M. smegmatis Housekeeping gene for qPCR analysis |
| **Msm_2758 qRvs sigA** | 5'-GAATCTCGGTTAATGCGA-3' | M. smegmatis Housekeeping gene for qPCR analysis |
| **Msm qFwd ctpC** | 5'-CGATCGGCTGTACCGGTTGATCCGGGATCAG-3' | M. smegmatis ctpC gene for qPCR analysis |
| **Msm qRvs ctpC** | 5'-CGATCGGCTGTACCGGTTGATCCGGGATCAG-3' | M. smegmatis ctpC gene for qPCR analysis |
Table 2. Free metal ATPase activity kinetic parameters of *M. tuberculosis* and *M. smegmatis* CtpC

| Metal | *M. tuberculosis* CtpC | *M. smegmatis* CtpC |
|-------|-----------------------|---------------------|
|       | $V_{\text{max}}$ (μmol/mg/h) | $K_{1/2}$ (nM) | $V_{\text{max}}$ (μmol/mg/h) | $K_{1/2}$ (nM) |
| Mn$^{2+}$ | 1.1 ± 0.2$^a$ | 0.009 ± 0.008 | 1.06 ± 0.06 | 0.005 ± 0.002 |
| Zn$^{2+}$ | 0.30 ± 0.01 | 0.019 ± 0.001 | 0.38 ± 0.07 | 0.02 ± 0.01 |

$^a$ Errors for $V_{\text{max}}$ and $K_{1/2}$ are asymptotic standard errors reported by the fitting software Kaleidagraph (Synergy).
CtpC a mycobacterial Mn$^{2+}$-ATPase

### Table 3. *M. tuberculosis* *ctpC::hyg* mutant accumulates Mn$^{2+}$

| Metal | Whole cell metal content (pmol/mg protein) | 7H9 media | Complemented 7H9 media + Zn$^{2+}$ |
|-------|-------------------------------------------|-----------|----------------------------------|
|       | Wild type | *ctpC::hyg* |                              |
| Mn    | 1.2 ± 0.3 | 2.1 ± 0.1* | 1.7 ± 0.1                      |
| Zn    | 317 ± 12  | 283 ± 5   | 314 ± 23                       |
| Cu    | 28 ± 6    | 25 ± 1    | 28 ± 3                         |
| Co    | 0.31 ± 0.04 | 0.23 ± 0.02 | 0.26 ± 0.06                  |
| Fe    | 237 ± 38  | 192 ± 7   | 455 ± 13                       |
| Zn    | 654 ± 18  | 581 ± 35  | 689 ± 28                       |

1Cells were grown to log phase

*Statistical significance at p<0.037*
TABLE 4. Secreted fraction metal content of *M. tuberculosis* strains

| Metal | Secreted fraction metal content (pmol/mg protein) |
|-------|--------------------------------------------------|
|       | Wild type | *ctpC::hyg* | Complemented |
| Mn    | 15.7 ± 0.9 | 9.2 ± 0.5 | 22 ± 1 |
| Zn    | 105 ± 2 | 91 ± 21 | 99 ± 2 |
| Cu    | 38 ± 10 | 36 ± 10 | 30 ± 7 |
| Fe    | 605 ± 8 | 583 ± 20 | 613 ± 12 |

1Cells were cultured on Sauton’s media supplemented with 50 μM Mn2+.
### TABLE 5. *M. smegmatis* cytosolic and secreted fraction metal levels

| Metal | Cytosolic fraction (pmol/mg protein) | Secreted fraction (pmol/mg protein) |
|-------|--------------------------------------|-------------------------------------|
|       | Wild type | *ctpC::hyg* | Complemented | Wild type | *ctpC::hyg* | Complemented |
| Mn    | 7.1 ± 0.9 | 20 ± 1     | 12 ± 2       | 38 ± 3     | 20 ± 4     | 41 ± 2       |
| Zn    | 596 ± 17  | 519 ± 47   | 492 ± 67     | 182 ± 13   | 162 ± 24   | 168 ± 34     |
| Cu    | 8 ± 2     | 4 ± 1      | 9 ± 3        | 22 ± 4     | 14 ± 2     | 27 ± 3       |
| Fe    | 489 ± 24  | 457 ± 59   | 480 ± 43     | 629 ± 36   | 784 ± 45   | 617 ± 28     |

1Cells were cultured on Sauton’s media supplemented with 50 μM Mn^{2+}. 

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*CtpC a mycobacterial Mn^{2+}-ATPase*
Table 6. Peptides identified by LC/MS corresponding to Sod from *M. tuberculosis* and *M. smegmatis*

| Strain                  | Identified peptides                                      | Protein                                      | Accession number |
|-------------------------|----------------------------------------------------------|----------------------------------------------|------------------|
| *M. tuberculosis*       | (K)AFWNVNVWADVQSR(F)                                     | SodA superoxide dismutase [Fe].              | NP_218363        |
| Wild type               | (K)EDHSAILLNK(F)                                         | *Mycobacterium tuberculosis* H37Rv           |                  |
|                         | (K)YAATSQTK(F)                                           |                                              |                  |
|                         | (K)NLSPNGGDKPTGELAAADAFGSFDK(F)                          |                                              |                  |
|                         | (K)AKEDHSAILLNK(F)                                       |                                              |                  |
| *M. tuberculosis*       | (K)AFWNVNVWADVQSR(F)                                     | SodA superoxide dismutase [Fe].              | NP_218363        |
| ctpC::hyg               | (K)YAATSQTK(F)                                           | *Mycobacterium tuberculosis* H37Rv           |                  |
|                         | (K)AKEDHSAILLNK(F)                                       |                                              |                  |
|                         | (K)EDHSAILLNK(F)                                         |                                              |                  |
| *M. smegmatis*          | (K)AFWNVNVWDDVQNR(F)                                     | Msmeg_6427 superoxide dismutase [Mn].        | ABK71950         |
| Wild type               | (K)NKSPNGGDKPTGELAAADQFGSFDK(F)                          |                                              |                  |
| ctpC::hyg               | (K)AFWNVNVWDDVQNR(F)                                     | Msmeg_6427 superoxide dismutase [Mn].        | ABK71950         |
A novel $P_{1B}$-type $Mn^{2+}$ transporting ATPase is required for secreted protein metallation in mycobacteria

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