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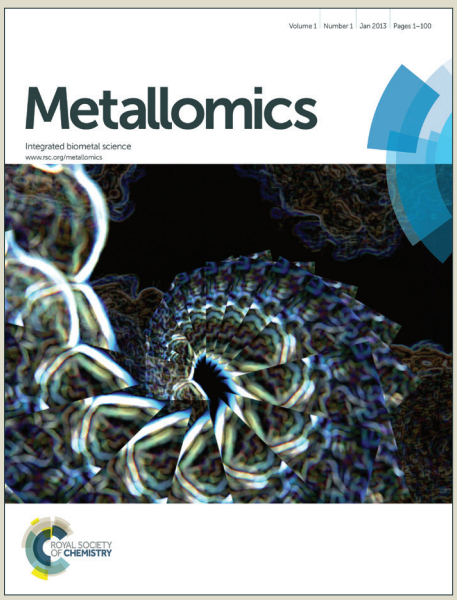
Accepted Manuscript



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Copper Homeostasis in *Mycobacterium tuberculosis*

Xiaoshan Shi and K. Heran Darwin*

Keywords: *Mycobacterium tuberculosis*, copper resistance, virulence

New York University School of Medicine

Department of Microbiology

550 First Avenue MSB 236

New York, NY, 10016, USA

*To whom correspondence should be addressed:

E-mail: heran.darwin@med.nyu.edu

Phone: (212) 263-2624

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Abstract

Copper (Cu) is a trace element essential for the growth and development of almost all organisms, including bacteria. However, Cu overload in most systems is toxic. Studies show Cu accumulates in macrophage phagosomes infected with bacteria, suggesting Cu provides an innate immune mechanism to combat invading pathogens. To counteract the host-supplied Cu, increasing evidence suggests that bacteria have evolved Cu resistance mechanisms to facilitate their pathogenesis. In particular, *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis, has evolved multiple pathways to respond to Cu. Here, we summarize what is currently known about Cu homeostasis in *Mtb* and discuss potential sources of Cu encountered by this and other pathogens in a mammalian host.

Mtb: a successful intracellular human pathogen

Mtb infects nearly one-third of the world's population and kills 1.3 million people annually, making it one of the most devastating infectious agents on earth (http://www.who.int/tb/publications/global_report/en/). Tuberculosis is transmitted by the inhalation of aerosolized droplets containing *Mtb* bacilli coughed or sneezed by an infected person. Once bacteria are inhaled, they localize to the alveoli, where macrophages and dendritic cells phagocytose them. *Mtb* has evolved to survive within macrophages that have multiple antimicrobial activities, including the production of reactive nitrogen and oxygen intermediates, phagosomal acidification and iron limitation.[1-3] Numerous labs around the world are working to understand how *Mtb* is able

to respond to this hostile environment, the knowledge of which may help in the development of improved tuberculosis treatments.

Evidence for Cu in the host response to mycobacterial infections

Cu is essential for the development of almost all aspects of mammalian physiology, thus defects in Cu homeostasis almost certainly impact immune responses to microbial infections. Dietary Cu-deficiency in farm animals is linked to a higher incidence of bacterial infections,[4] perhaps because Cu-deficient diets reduce the number of antibody-producing cells in mice.[5] In a study that was key to the realization that Cu and other metals may be important for impacting the outcome of tuberculosis infections, Bermudez and coworkers found that the concentration of Cu markedly increases in phagolysosomes of peritoneal-derived mouse macrophages after infection with several *Mycobacterium* species.[6] In a later study, it was found that dietary supplementation with Cu results in the accumulation of Cu in lung granulomas of *Mtb*-infected guinea pigs, and coincides with a reduction in bacterial burden.[7] Collectively, these data suggest that Cu is mobilized in mammals to control bacterial growth.

Further supporting these observations, several studies show a link between Cu transporting machinery and antimicrobial activity. Petris and co-workers showed Cu enhances the bactericidal activity of RAW264.7 murine macrophage cells toward *E. coli* . Treatment of RAW264.7 macrophages with proinflammatory factors, such as IFN-γ or lipopolysaccharide, is associated with increased levels of the high affinity Cu importer CTR1 at the plasma membrane[8] and the P_{1B}-type ATPase ATP7A.[9] P-type ATPases

are integral membrane proteins that use ATP to either transport molecules in or out of a cell and are found in both eukaryotes and prokaryotes.[10] Significantly, ATP7A is trafficked from the trans-Golgi network into the phagosomal compartment of IFN-γ activated macrophages, providing a possible mechanism by which bactericidal levels of Cu may be delivered into phagolysosomes.[9] Moreover, IFN-γ stimulated RAW264.7 macrophages kill *E. coli* lacking the Cu efflux pump CopA more effectively than wild type bacteria, and this effect is reduced by silencing the ATP7A gene.[9] These data show that mammalian Cu transporters can mobilize Cu into phagosomal compartments to control the growth of at least one bacterial species. It remains to be determined if macrophage-associated ATP7A is also required for controlling mycobacterial or other infections.

Copper is both essential and toxic for *Mtb*

Although Cu is antimicrobial, it is also essential. Cu can undergo reversible oxidation states between reduced Cu^{+} and oxidized Cu^{2+} and has a high redox potential, making it a critical cofactor of enzymes used for electron transfer reactions in the presence of oxygen. In *Mtb*, the most prominent Cu binding enzymes include cytochrome *c* oxidase and the Cu/Cu superoxide dismutase[11], which contributes to resistance to oxidative stress.[12] Thus, like for most life forms, Cu is essential for *Mtb* viability.

Of course, too much Cu is toxic to *Mtb*.[7, 13, 14] Several mechanisms have been ascribed to the toxicity of Cu. Under aerobic conditions, Cu can react with hydrogen

peroxide (H_2O_2) to create hydroxyl radical (•OH) and hydroxyl anion (OH^-) via a Fenton-like reaction:[15]

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^- + \bullet OH$$

These molecules can react with and irreversibly damage many macromolecules including proteins, lipids and nucleic acids, potentially leading to cell death.[16] However, it has not yet been definitively proven that the production of these reactive products is the mechanism of Cu-induced cytotoxicity in any bacterial system. In fact, Imlay and colleagues showed that a mutant *E. coli* strain that hyper-accumulates Cu is more resistant to H_2O_2 stress than bacteria without accumulated Cu.[17] Furthermore, Cu treatment is surprisingly associated with less, not more, oxidative DNA damage, even though the production of reactive oxygen species (ROS) is apparent in these bacteria.[17] Although Imlay and colleagues tested several hypotheses to explain the protective effects of Cu, they could not identify a mechanism to explain their observations. The authors of this study speculated that an alteration in Cu accumulation results in an adaptation that either sequesters or otherwise prevents Cu from interacting with hydrogen peroxide near DNA, a hypothesis that remains to be tested.[17]

Because the creation of ROS does not explain why Cu is toxic to *E. coli*, Macomber *et al* tested the hypothesis that Cu displaces iron-sulfur (Fe-S) clusters from important metabolic enzymes to inactivate bacterial growth.[18] Indeed, a study found Cu targets isopropylmalate dehydratase, which is needed for the synthesis of branched chain amino acids. An *E. coli* strain lacking several Cu homeostasis proteins is very Cu sensitive, but the addition of several branched-chain amino acids restores some growth

during Cu treatment.[18] Initially it was thought that Cu increases the amount of intracellular H_2O_2, which might directly inactivate several amino acid biosynthetic pathways; however, the presence of oxygen (and thus H_2O_2) is not needed to see this effect. Furthermore, growth inhibition occurs at the same time as the displacement of Fe atoms from the solvent-exposed cluster of dehydratases, suggesting that Cu inactivates these enzymes by liganding to coordinating S atoms.[18] Unlike what was observed in *E. coli*, the addition of branched-chain amino acids to a Cu susceptible *Salmonella* strain culture does not rescue Cu toxicity, suggesting the mechanisms of Cu toxicity may be multifactorial and vary from organism to organism.[19]

Another target of Cu toxicity has been discovered in the obligate human pathogen *Neisseria gonorrheae.* Cu is predicted to disrupt Fe-S clusters of the enzyme HemN, which is required for the heme biosynthesis. Failure to equip hemoproteins such as catalases, peroxideses and nitric oxide (NO) reductases with their cognate prosthetic group may lead to the increased toxicity of reactive species and thus decreased bacterial survival.[20]

Interestingly, Nathan and colleagues showed that NO can reduce Cu^{2+} to Cu^{+}.[21] Macrophages infected with *Mtb* and other pathogens stimulate the production NO, which can kill invading microbes by different mechanisms, including the production of reactive nitrogen and oxygen species, the displacement of metal co-factors from enzymes, and in ways that have yet to be identified.[22] Perhaps another mechanism of NO mediated toxicity is via the reduction of Cu to make it more toxic to invading microbes.

Copper homeostasis in *Mtb*: a tale of three pathways

Several independent lines of study converged upon the conclusion that *Mtb* expresses Cu resistance pathways in order to successfully persist in a mammalian host. Talaat and colleagues found the first Cu responsive operon in *Mtb* after following up on the identification of a unique genomic island called the *in vivo* expressed genomic island or iVEGI that is highly induced in bacteria isolated from mouse lungs but not in bacteria that are grown in broth culture.[23] Analysis of this island for potential transcriptional units uncovered the presence of an operon (Rv0967-Rv0970) including *ctpV*, a cation P-type ATPase that is predicted to transport Cu out of *Mtb* (Fig. 1).[24] Moreover, the operon is highly induced upon Cu treatment, therefore, it was named the copper sensitive operon (*cso*).[25] The organization of the genes in the *cso* operon is similar to that of the *cmt* (cadmium/lead metal transporter) operon, in which the first gene encodes a cadmium and lead-sensing transcriptional regulator, CmtR, controlling the whole operon.[26] Liu *et al* hypothesized the first gene in the operon, Rv0967, was a Cu responsive transcriptional regulator. Indeed, Rv0967, later named CsoR (copper sensitive operon repressor), binds to DNA as a dimer of dimers in the absence of Cu.[25] At elevated Cu^{+} concentrations the binding affinity of CsoR to the promoter of the *cso* operon is reduced due to a conformational change in the CsoR-DNA complex caused by Cu^{+}. Giedroc and colleagues concluded that this was the mechanism of derepression of the *cso* operon observed in Cu-treated *Mtb* cultures.[24]

In order to determine the contribution of the *cso* operon on Cu resistance, Talaat and colleagues characterized CtpV, a predicted P-type ATPase.[24] Overexpression of

the *Mtb cso* operon in *Mycobacterium smegmatis* (*M. smegmatis*), a non-pathogenic relative of *Mtb* that does not have a *cso* operon, shows lower levels of intracellular Cu accumulation suggesting CtpV functions as an efflux pump.[13] This study also showed that *ctpV* is the most highly Cu-induced *ctp* gene of 11 predicted *ctp* genes in the *Mtb* genome.[13] In vitro, an *Mtb ctpV* mutant is hyper-sensitive to Cu, and this phenotype can be partially complemented *in trans.* Mice infected with a *ctpV* mutant survive significantly longer than mice infected with wild type *Mtb,* however this effect cannot be rescued when *ctpV* is added back at another site on the chromosome. Furthermore, in guinea pig infections, the numbers of *ctpV* mutant bacteria is significantly lower than those of wild type bacteria at 21 days after infection, however, no colonization defect is observed at a later time point.[13] Taken together, CtpV appears to be required for resistance of Cu toxicity, but its contribution to *Mtb* virulence is unclear.

In another study, Nathan and colleagues discovered the first metallothionein in *Mtb* named MymT (mycobacterial metallothionein).[21] Metallothioneins are small, cysteine-rich proteins with the ability to bind metal ions. Their biological roles include metal detoxification, intracellular distribution and defense against oxidative stress.[27] MymT harbors several cysteines (Cys) arranged in Cys-X-Cys or Cys-X-His motifs that allow it to coordinate metal ions.[21] Expression of *mymT* is highly induced by several metals including Cu, cadmium, cobalt, nickel and zinc, with the strongest induction by Cu.[21] Mass spectrometry analysis shows that MymT can bind with up to six Cu^{+} ions in a solvent-shielded thiolate core.[21] Although deletion of *mymT* from *Mtb* leads to

increased Cu sensitivity in vitro, MymT does not appear to be essential for virulence in a murine infection model.[21]

Several years after the discovery of CsoR, a second locus required for Cu resistance was characterized. Mycobacterial copper transport protein B (MctB/Rv1698) (Fig. 1) was initially identified as a putative outer membrane protein by a genome-wide secondary structure prediction study of *Mtb.*[28] Mycobacteria do not have canonical outer membranes as observed in Gram-negative bacteria but instead have what is termed a "mycomembrane", which is composed of mycolic acids and other components unique to mycobacteria.[29] Like Gram-negative bacteria, mycobacteria have what appear to be porins,[30-33] thus it was predicted that MctB might have this function.[34] Deletion of *mctB* or its homologue in *M. smegmatis*, ms3747, leads to Cu accumulation in the bacterial cytoplasm and increased Cu sensitivity, demonstrating an essential role of MctB in maintaining low Cu concentrations in mycobacteria.[7]

Niederweis and colleagues used two models to test the role of MctB during infections. In mice, an *Mtb mctB* mutant is modestly attenuated compared to wild type *Mtb*.[7] It was presumed that Cu would be more effective if it encountered *Mtb* in a hypoxic environment, where Cu^{+} would remain reduced. The authors of this study noted that mice do not form hypoxic granulomas like humans, and that guinea pigs represent a better model for human infections.[35] Wolschendorf *et al* tested the fitness of an *mctB* deletion mutant in both animal models. In BALBc mice, wild type *Mtb* grows to about 10 times higher numbers than an *mctB* mutant. This difference is further exacerbated by the addition of Cu sulfate ($CuSO_4$) to the animals' drinking water, suggesting the

attenuation is due to a Cu specific effect.[7] In guinea pigs, the difference growth defect between the wild type and *mctB* strains is more pronounced than what is observed in mice.[7] This may be consistent with the notion that a more hypoxic environment may more effectively use Cu to control bacterial growth.

Although MctB was initially described as a putative mycomembrane protein, recent studies suggest MctB may be anchored to the inner membrane.[36] Thus it is less clear that MctB forms a channel to export Cu and may have a different activity to maintain Cu homeostasis in mycobacteria. Furthermore, because *mctB* expression is not Cu inducible,[24] it may have functions that are important for reasons beyond Cu resistance.

The final pathway to be involved in Cu homeostasis was identified in an attempt to understand the link between proteasome function and *Mtb* pathogenesis. The *Mtb* proteasome, and ATP-dependent chambered protease, is required for causing lethal infections in animals for reasons that have largely been mysterious.[37-40] Using microarray analysis, Festa *et al* tested the hypothesis that the proteasome impacts transcriptional regulation in *Mtb*.[14] The analysis identified a new regulon unique to *Mtb* that includes *lpqS* (encoding a putative lipoprotein), *mmcO* (encoding a mycobacterial multicopper oxidase), Rv2963 (encoding a possible permease), *mymT*, *socAB* (small ORF induced by copper A and B), and *ricR*, which encodes a homologue of CsoR.[14] All genes are Cu-inducible suggesting RicR represses gene expression under Cu-deplete concentrations (Fig. 1).[14, 21, 24]

A *ricR* null mutant, which overexpresses all genes in the RicR regulon, is hyper-resistant to Cu in vitro, suggesting that one or more RicR-regulated genes are important in combating Cu toxicity.[14] The only RicR-regulon gene products that have known functions are MmcO, MymT and RicR itself. MmcO can oxidize Fe^{2+} to Fe^{3+} and perhaps also convert toxic Cu^{+} into Cu^{2+}.[41] Although MmcO is required for Cu resistance in vitro, it alone does not significantly contribute to *Mtb* virulence in a mouse model of infection.[41, 42] Similarly, deletion and/or disruption of any single RicR-regulated gene or even simultaneous deletion of the two major Cu-protective genes (*mymT* and *mmcO*) has a minimal impact on the growth of *Mtb* in mice at time points of up to eight weeks after infection.[42] However, an *Mtb* strain with a mutant allele of RicR that cannot respond to Cu and constitutively represses all of the genes of the RicR regulon is highly sensitive to Cu and attenuated for growth in mice.[42] Taken together, it appears that deletion of two or more RicR-regulated genes will be necessary to observe a robust in vivo phenotype. Alternatively, the contribution of individual RicR-regulated genes may need to be ascertained in an infection model more closely resembling that of humans.

Conclusions and Prospects

It is notable that deletion of any single gene associated with Cu resistance, with the exception of *mctB*, does not significantly affect *Mtb* virulence, perhaps suggesting that the loss of one Cu-responsive gene may be compensated by the induction of expression of another. It is also worth mentioning that all of the above systems lack a key component: some Cu chaperone. Cu is assumed to always bind to proteins or

otherwise be liganded to small molecules, thus it remains to be determined how Cu is transferred to the various Cu binding proteins and efflux pumps described here. It is tempting to speculate that there may be one or more Cu chaperones shared by all three systems that are critical for their function. It is also possible that some thiol-containing small molecule, such as mycothiol,[43] the major thiol found in mycobacteria, may perform a Cu exchange function given its high affinity for Cu^{+}.[44] However, it is hard to envision how this could be regulated in a controlled manner. In addition to the search for a Cu chaperone, numerous questions still need to be addressed. For example, how does Cu kill *Mtb*? What are the sources of Cu during a tuberculosis infection? Ceruloplasmin is a serum ferroxidase that contains more than 95% of the Cu found in plasma.[45] It is tempting to speculate that ceruploplasmin might play a critical role in Cu mobilization and antimicrobial activity. Can Cu resistance mechanisms in bacteria be targeted for drug development? A recent high-throughput drug screen identified compounds that possess Cu-dependent anti-*Mtb* activities, the mechanisms of which have not been elucidated.[46] Intriguingly, another screen for compounds that inhibit the ability of *Mtb* to kill cultured cells found a small molecule, a benzyloxybenzylidene-hydrazine compound called BBH7, which strongly induces the RicR regulon.[47] BBH7 was selected for analysis because it inhibits the secretion of a major virulence protein called EsxA.[47] Based on these studies, it is tempting to speculate that there is a link between Cu homeostasis and virulence protein secretion in *Mtb*.

Besides mycobacteria, can Cu also work as an antimicrobial weapon against other pathogens such as fungi, viruses, or eukaryotic parasites? Cu acquisition and

detoxification pathways have been implicated in the virulence of the fungal pathogen *Cryptococcus neoformans*, showing Cu may also be used by the innate immune system to battle fungi.[48] Further investigations into the biochemistry, genetics, and physiology of Cu homeostasis in both the host and pathogen will be essential to have a better understanding of the role of Cu in tuberculosis and other infectious diseases.

Acknowledgements

Copper research in the Darwin laboratory is supported by NIH grant HL92774.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Figures legend:

Fig. 1. Model of Cu-mediated control of *Mtb* in activated macrophages.

Upon activation, Cu uptake in macrophages is increased due to the elevated levels of the Cu importer CTR1 and ATP7A. ATP7A traffics to the phagosome, potentially leading to the increased concentration of Cu in that compartment. To combat the toxicity of excess Cu, *Mtb* has at least three independent Cu resistance pathways as described in the text. The Cu-binding ferroxidase ceruloplasmin[49, 50] may contribute a source of Cu to control *Mtb* and other infections.

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