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Resistance mechanisms of *Mycobacterium tuberculosis* against phagosomal copper overload

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SUMMARY

Mycobacterium tuberculosis is an important bacterial pathogen with an extremely slow growth rate, an unusual outer membrane of very low permeability and a cunning ability to survive inside the human host despite a potent immune response. A key trait of *M. tuberculosis* is to acquire essential nutrients while still preserving its natural resistance to toxic compounds. In this regard, copper homeostasis mechanisms are particularly interesting, because copper is an important element for bacterial growth, but copper overload is toxic. In *M. tuberculosis* at least two enzymes require copper as a cofactor: the Cu/Zn-superoxide dismutase SodC and the cytochrome *c* oxidase which is essential for growth *in vitro*. Mutants of *M. tuberculosis* lacking the copper metallothionein MymT, the efflux pump CtpV and the membrane protein MctB are more susceptible to copper indicating that these proteins are part of a multipronged system to balance intracellular copper levels. Recent evidence showed that part of copper toxicity is a reversible damage of accessible Fe-S clusters of dehydratases and the displacement of other divalent cations such as zinc and manganese as cofactors in proteins. There is accumulating evidence that macrophages use copper to poison bacteria trapped inside phagosomes.

Here, we review the rapidly increasing knowledge about copper homeostasis mechanisms in *M. tuberculosis* and contrast those with similar mechanisms in *E. coli*. These findings reveal an intricate interplay between the host which aims to overload the phagosome with copper and *M. tuberculosis* which utilizes several mechanisms to reduce the toxic effects of excess copper.

Keywords

nutrient; uptake; bactericidal; efflux; homeostasis; immune defence

Copper is an important element for bacterial growth

The ability of copper ions to undergo reversible oxidation from Cu(I) to Cu(II) combined with its high redox potential makes copper an important cofactor in enzymes used for electron transfer reactions in the presence of oxygen[1]. Thus, it is widely believed that copper is an essential trace element required for survival by all organisms from bacteria to humans[2]. While copper homeostasis in *E. coli* is well studied, the corresponding proteins and mechanisms in mycobacteria remain largely uncharacterized. Because the environment in

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which *M. tuberculosis* spends most of its life cycle is the macrophage phagosome, a compartment of oxidative and nitrosative stress, understanding the homeostasis of redox active metals such as copper is of critical importance.

In *M. tuberculosis* at least two enzymes require copper as a cofactor: the Cu/Zn-superoxide dismutase SodC and the cytochrome *c* oxidase. Since superoxide anions cannot cross lipid membranes, bacteria use two superoxide dismutases to protect both the periplasm and the cytoplasm from oxidative stress: The periplasmic Cu/Zn-containing superoxide dismutase SodC protects against extracellular superoxide[3] or superoxide generated in the periplasm[4], while the Mn/Fe-containing superoxide dismutase SodA detoxifies superoxide in the cytoplasm[5]. The *sodC* mutant of *M. tuberculosis* is more susceptible to superoxide *in vitro* and to killing by activated macrophages[6] establishing that the Cu/Zn superoxide dismutase contributes to the resistance of *M. tuberculosis* against oxidative stress. However, *sodC* expression is reduced when *M. tuberculosis* switches from replicative to persistent infection states, suggesting that its activity is required only during the early stages of infection[7]

The cytochrome *c* oxidase is the most energy-efficient terminal oxidase and is used during exponential growth of *M. tuberculosis* in the presence of oxygen[8]. The genes encoding the three subunits of the cytochrome *c* oxidase CtaC, CtaD, CtaE (Rv2200c, Rv3043c, Rv2193) are essential for growth of *M. tuberculosis in vitro*[9]. The fact that copper is required for the function of the cytochrome *c* oxidase indicates that copper is an essential nutrient, at least in the exponential growth phase of *M. tuberculosis*.

Mechanism(s) of copper toxicity

Iron-sulfur cluster degradation

It has long been thought that copper toxicity is due to a Fenton-like reaction between Cu(I) and hydrogen peroxide, producing hydroxyl radicals which then induce oxidative stress inside cells and cause DNA damage[10]. Indeed, DNA damage has been observed in eukaryotic cells upon copper exposure[11] and prokaryotic cells respond to copper stress by inducing the reactive oxygen species (ROS) stress response[12, 13]. However, recent work in *E. coli* and *Bacillus subtilis* suggests an alternate mechanism of copper toxicity. Macomber *et al*. showed that a copper accumulating *E. coli* mutant is more sensitive to copper stress during anaerobic growth than aerobic growth, that excess copper actually protects the mutant from hydrogen peroxide-mediated cellular damage and that copper accumulation does not increase the rate of hydroxyl radical production, leading the authors to conclude that Fenton-like chemistry does not occur upon copper accumulation in *E. coli* cells[14]. Indeed, even wild-type *E. coli* is more sensitive to copper under anaerobic conditions than when grown in the presence of oxygen[15]. It also has been shown that copper reversibly damages accessible Fe-S clusters of dehydratases in *E. coli*, while proteins with buried Fe-S clusters are not subject to copper damage[10]. It has been proposed that copper converts $[^{4}Fe\text{-}^{4}S]^{2+}$ to $[^{3}Fe\text{-}^{4}S]^{0}$ clusters which are then further degraded by an unknown mechanism[10]. In *B. subtilis*, copper-stress induces expression of genes involved in iron acquisition and Fe-S cluster biogenesis, but only a few genes responding to reactive oxygen intermediates are induced[16]. The role of dehydratases in metabolism may explain why excess copper reduces the metabolic capacity of cells[10, 16].

Metal cofactor replacement

Copper also is able to replace other metals which act as cofactors in proteins. For example, Cu(II) can replace Zn(II) in the active site of human glyoxalase I[17] and in zinc-finger domains of human estrogen receptor protein[18], thereby inactivating these proteins. In *M. tuberculosis*, copper stress greatly induces the expression of *cadI*, encoding a putative glyoxalase I, which likely contains zinc[12]. The authors hypothesized that replacement of

zinc by copper resulted in inactive enzyme and a feedback loop that upregulates *cadI* expression[12]. Deletion of the gene encoding the multicopper oxidase CueO from *E. coli* results in copper accumulation and growth defects under copper stress, both of which can be reduced by the addition of other transition metals (Zn(II), Mn(II), Fe(III))[19]. These results suggest that metal cofactor replacement is also a mechanism of copper toxicity.

No free copper in the cytoplasm

Bacteria respond quickly to increased cellular copper by increasing expression of efflux pumps as well as copper chaperones and multicopper oxidases[15, 20, 21]. Therefore, it is unlikely that there is free copper in bacterial cells which would be available to create reactive oxygen species under standard growth conditions. In *E. coli*, under standard conditions, the cellular copper concentration is approximately 10 μM[21]. However, the copper sensing transcriptional regulator CueR responds to zeptomolar concentrations of Cu(I), leading authors to conclude there is no free copper in the cytosol of prokaryotic cells[22, 23]. In *E. coli*, mutation of *dsbC*, a periplasmic disulfide bond isomerase, which resolves incorrect disulfide bonds, results in copper sensitivity[24]. A possible explanation for this observation is that excess copper in the periplasm leads to incorrect disulfide bond formation. Thus, loss of functional DsbC may result in increased copper sensitivity[24]. However, direct experimental evidence for free copper in the periplasm of *E. coli* is lacking.

Role of copper in the host immune response

Copper is required for the development and maintenance of the mammalian immune system

Several lines of evidence indicate that copper is required for both innate and adaptive immune responses. Human infants with Menkes disease, which causes copper deficiency, are more susceptible to infection[25]. Increasing the amount of copper in the diets of malnourished children reduces the incidence of infectious diseases[26]. Mice raised on a copper-depleted diet produce fewer antibody-producing cells than those fed a normal diet and show increased morbidity upon infection[27, 28]. Copper-deficient diets reduce the respiratory burst and subsequent microbicidal activities of neutrophils and macrophages as well as T-cell dependent antibody production[29 and references therein]. We have shown that increasing the dietary copper of *M. tuberculosis*-infected mice reduces the number of lymphocytic infiltrates and lung lesions[30]. Additionally, copper accumulates in granulomas isolated from *M. tuberculosis*-infected guinea pigs compared to un-affected lung tissue indicating that copper is utilized by the immune system to fight the infection[30]. These results show that copper is required not only for the functional development of the animal as a whole, but also for the multiple cell types that make up the host immune system and that copper may be used directly as a host defense mechanism.

Macrophages take up copper in response to mycobacterial infection

M. tuberculosis elicits multiple immunological responses upon infection of the human host. Macrophages are recruited to the infection site, and the bacteria are phagocytosed. *M. tuberculosis* interrupts the normal phagocytosis program by blocking phagosome-lysosome fusion, without which the phagosome cannot acidify. Hence, the pH within the *M. tuberculosis*-containing phagosome remains near 6.4 (Fig. 1C) (reviewed in[31]). When the macrophage is activated by interferon-γ (IFN-γ) phagosome maturation proceeds as normal and the vacuole acidifies to a pH of 4.5 – 5.0 (Fig. 1CD)[32, 33]. When infected with *Mycobacterium avium*, macrophage phagosomes significantly accumulate copper in an IFN-γ dependent manner[34] (Fig. 1D). Infected macrophages left unactivated or activated with TNF-α do not significantly accumulate copper in bacteria-containing phagosomes[34]. Whether the increased copper acts directly or indirectly to kill *M. tuberculosis* is unclear.

Copper could be used to generate oxidative stress within the phagolysosome, or it could be taken up by bacteria directly and exert toxic effects as described above. Additionally, it was recently shown that release of zinc into phagolysosomes increases bactericidal activity of macrophages[35], indicating that other heavy metals may be used in a manner similar to copper as antibacterial agents.

ATP-driven copper-transport is required for bactericidal activity of macrophages

The role of copper in aiding macrophage bactericidal activity was further investigated by Petris and co-workers. Addition of copper to IFN-γ activated macrophages increases their bactericidal activity against *E. coli*[36]. Activation of human macrophages with IFN-γ or LPS results in a dose-dependent increase in copper uptake and increased expression of the copper transport protein, CTR1, as well as the copper-transporting ATPase, ATP7A[36]. Under normal growth conditions CTR1 is mainly localized on the cytoplasmic membranes of eukaryotic cells, while ATP7A is associated with the trans-Golgi network (TGN)[37]. ATP7A remains associated with the TGN under low copper conditions where it transports copper into the TGN lumen for incorporation into copper-requiring proteins (Fig. 1A), but is trafficked to the cytoplasmic membrane under high copper to excrete excess copper (Fig. 1B)[38]. However, protein and mRNA levels remain unchanged in response to copper, showing that the mechanism of ATP7A response to copper stress is post-translational[39]. Trafficking of ATP7A from the TGN to post-Golgi vesicles is dependent on the monomeric copper chaperone ATOX1, which binds a single copper atom[38, 40, 41] (Fig. 1A). Additionally, vesicles containing ATP7A are trafficked to the phagosome when macrophages are stimulated with IFN-γ and exposed to excess copper[36]. ATP7A also is required for killing of *E. coli* in infected macrophages[36]. *E. coli* lacking the copper efflux pump, CopA, is more susceptible to macrophage killing than wild-type *E. coli*. This phenotype is rescued by RNAi-mediated knockdown of the *ATP7A* gene[36]. From these data, a model for the role of copper in controlling *M. tuberculosis* infection of macrophages can be proposed (Fig. 1CD). Upon infection, *M. tuberculosis* prevents phagolysosome fusion and therefore acidification of the bacteria-containing compartment. Once the macrophages are activated by IFN-γ phagolysosome fusion proceeds, expression of *CTR1* and *ATP7A* is increased, ATP7A protein is trafficked to the phagolysosome, and the copper concentration of the compartment increases. Taken together, these results reveal an important role for copper in macrophage-mediated killing of bacteria.

Copper resistance and virulence of pathogenic bacteria

Considering the importance of copper for the immune system of mammals it is not surprising that copper resistance is required for full virulence of pathogenic bacteria. For example, *E. coli* mutants deficient in copper efflux are more susceptible to killing by macrophages[36]. The copper-transporting P-type ATPases of *Salmonella enterica sv. Typhimurium* (*S. Typhimurium*) are required for survival in macrophages[42], and in *Listeria monocytogenes* for persistence in the liver during murine infection[43]. The Cu-efflux pumps CopA1 and CopA2 of *Pseudomonas aeruginosa* are required to establish infection in an *Arabidopsis thaliana* model system[44]. Mutation of the multi-copper oxidase gene *cueO* of *S. Typhimurium* results in attenuated mouse colonization, but does not increase susceptibility to macrophage killing[45]. It appears that copper efflux alone is not sufficient to prevent copper toxicity in bacteria; multicopper oxidases and perhaps other copper binding proteins may also be required.

Copper homeostasis in *M. tuberculosis*

In the last few years several mechanisms of copper homeostasis have been identified in *M. tuberculosis*[30, 46–49]. Here, we will discuss these mechanisms of *M. tuberculosis* in detail (Fig. 2A).

Copper import

The mechanism of copper import awaits characterization in *M. tuberculosis*; indeed copper influx has not been described even for the most-studied organisms such as *E. coli*. It is unknown if there are dedicated copper import proteins in *M. tuberculosis*. Considering the very low permeability of cations across any lipid membrane[50], direct diffusion of copper ions across the mycobacterial outer and inner membranes is unlikely. However, heavy metal import proteins have been characterized in other bacteria. For example, overexpression of *hmtA* of *Pseudomonas aeruginosa* results in copper hypersensitivity and copper accumulation in *E. coli*[51]. Putative copper import proteins have been proposed in *Enterococcus hirae* and *B. subtilis*, but copper import has not been shown directly[20]. In *E. hirae copA* is induced under copper-limiting conditions and the deletion mutant is unable to grow on copper limiting media indicating that this gene encodes a copper uptake protein[52]. Similarly, in *B. subtilis*, *ycnJ* is induced under low copper conditions and the knock-out strain has a growth defect on copper-deficient media[53].

Copper efflux - CtpV and its regulator CsoR

The proteins involved in copper efflux are well characterized in *E. coli*[54](see below and Fig. 2B) and *E. hirae*[20], but have not been studied in *M. tuberculosis* until recently. The first hints to the *M. tuberculosis* copper-stress response came from mouse infection studies. Expression of an operon comprising genes *rv0967-rv0970* was induced during infection of mice compared to growth of *M. tuberculosis in vitro*[55]. The operon contains *ctpV* (*rv0969*), a putative cation transport P-type ATPase predicted to transport copper[56]. R*v0968* and *rv0970* encode proteins of unknown function (Fig. 2A). The operon was induced under copper stress *in vitro* and was named copper-sensitive operon (*cso*)[47]. Copper highly induced expression from the *cso* promoter compared to other metals indicating that the operon is copper specific and not part of a general metal-ion response pathway[47]. Because the structure of *cso* is reminiscent of the *cmt* (cadmium/lead-responsive) operon, in which the first gene encodes a regulator for the operon, the authors hypothesized the protein product of *rv0967* might also be a transcriptional regulator. Indeed, purified Rv0967 protein (renamed CsoR, copper sensitive operon regulator) specifically binds a pseudopalindromic sequence overlapping the *cso* promoter. Addition of copper to the CsoR-DNA complex releases the regulator from its cognate DNA sequence[47]. Furthermore, apo-CsoR binds equimolar amounts of Cu(I) which is coordinated by two cysteines and a histidine[47].

The characterization and structure of CsoR revealed that this protein represents a previously unknown class of copper-responsive regulators, which is much more widely distributed among prokaryotes than the well-characterized examples from *E. coli* (CueR) or *E. hirae* (CopY)[47]. Apo-CsoR represses transcription of *cso* and loses affinity to its operator upon copper binding, allowing transcription of the operon (Fig. 2A). In *B. subtilis*, CsoR negatively regulates expression of *copZ* and *copA* encoding a copper chaperone and a copper efflux pump, respectively, in a manner similar to *M. tuberculosis*. However unlike *M. tuberculosis*, *csoR* is transcribed separately from its target genes in *B. subtilis*[57]. Global transcriptional analysis by microarray experiments showed that CsoR regulates only its own promoter and hence expression of *cso* in *M. tuberculosis*[49].

The role of CtpV in the *M. tuberculosis* copper-stress response was further studied because CtpV is predicted to be a copper-transporting ATPase and *ctpV* expression is induced by elevated levels of copper[12]. *M. tuberculosis* CtpV has significant sequence similarity to CopA of both *E. coli* and *E. hirae*. Importantly, motifs conserved among metal-transport P-type ATPases are found in *M. tuberculosis* CtpV[12]. However, unlike the CopA proteins, CtpV lacks an apparent metal binding domain. Whether and how this alteration affects the activity of CtpV or its interactions with other proteins, such as copper chaperones, is unknown[12]. Deletion of *ctpV* from *M. tuberculosis* H37Rv does not give a general growth defect, but results in increased copper sensitivity compared to wild-type[46]. Moreover, expression of the *cso* in *M. smegmatis* decreases copper accumulation compared to wild-type *M. smegmatis*, indicating that *cso* mediates copper resistance by efflux[46]. Taken together, the copper-induced expression of *ctpV*, the similarity of CtpV to known copper efflux pumps, and its ability to reduce the copper burden of *M. smegmatis*, suggest that CtpV indeed functions as a copper efflux pump in *M. tuberculosis* (Fig. 2A). However, transport of copper by CtpV has yet to be demonstrated directly.

The *M. tuberculosis* genome encodes 11 putative cation transport P-type ATPases, three of which are predicted to transport Cu (CtpV, CtpA and CtpB)[12, 56]. Only expression of *ctpv* and *ctpG* is induced by copper. C*tpG* expression is further upregulated by copper in the absence of *ctpV*, suggesting CtpG may functionally compensate for the loss of CtpV[46]. Additionally, two predicted permease genes of unknown function, *rv0849* and *rv2963*, are induced by copper[12]; the role of these proteins in the copper response remains uncharacterized.

Expression of *ctpV* is induced during infection of mice compared to *in vitro* growth[55] and copper resistance is a known virulence determinant in several pathogenic bacteria (reviewed above) suggesting CtpV plays a role in *M. tuberculosis* pathogenesis. In one guinea pig infection experiment deletion of *ctpV* resulted in decreased colony forming units (CFUs) in the lung compared to wild-type *M. tuberculosis* 21 days after infection, but this difference was resolved later[46]. Additionally, the *ctpV* deletion mutant did not show any virulence defect in mice. Mouse survival was greatly increased compared to wild-type infection, but was not complemented by expression of *ctpV*, raising doubts whether this phenotype resulted from deletion of *ctpV*[46]. Taken together, these experiments suggested that CtpV is not required for colonization or proliferation[46].

Copper efflux - MctB (Rv1698)

Initial experiments showed that Rv1698 is a membrane protein, is surface-accessible, increases uptake of glycerol in a *M. smegmatis* porin mutant, and forms pores in artificial lipid bilayers[58, 59]. Additionally we found that mutation of *rv1698* in *M. tuberculosis* or its homolog in *M. smegmatis*, *ms3747*, resulted in increase of total cellular copperand increased the copper sensitivity of these mutants. These findings led us to suggest that Rv1698 was a pore-forming outer membrane protein involved in copper efflux[30]. However, additional experiments did not confirm surface accessibility of Rv1698 and indicated that it might be anchored in the inner membrane (manuscript in preparation, Fig. 2A). Copper accumulation by *M. smegmatis* lacking *ms3747* has been verified by inductively coupled plasma mass spectrometry (ICP-MS) (manuscript in preparation). It was confirmed that Ms3747 and Rv1698 are required for copper resistance, but it is unclear whether these proteins play a direct role in copper efflux. Alternatively, loss of Rv1698 may change the outer membrane so that more copper enters the periplasm and/or the cytoplasm of *M. tuberculosis*. We are currently performing experiments to identify the exact role of Rv1698 in copper resistance. The observation that *rv1698* expression is not induced by copper stress[12], but is induced during starvation[60] is consistent with a more pleiotropic function of Rv1698. Rv1698 is required for full virulence in mouse and guinea pigs[30], however, it is unclear whether the

virulence defect of the rv1698 mutant of *M. tuberculosis* is directly caused by its increased copper susceptibility.

The copper metallothionein MymT of *M. tuberculosis*

Metallothioneins (MTs) are characterized by their small size (<10kD) and cysteine-rich sequences with few hydrophobic residues. To date, only a few MTs have been described in bacteria, but many examples are present in eukaryotes[61, 62]. Metallothioneins typically bind 4–12 heavy metal ions in a solvent-shielded thiolate core[61]. The precise biological functions of MTs have yet to be defined; they certainly serve as metal ion sinks, but may have other functions as well. It has been proposed that MTs may function as metal chaperones, delivering metal ions to proteins that require them as cofactors[63]. Furthermore, it has been shown in *S. cerevisiae* that a copper loaded MT can be transported out of the cell to aid in copper-detoxification[64]. MTs, therefore, play an important role in copper homeostasis and detoxification in many cell types.

The mycobacterial metallothionein MymT was first identified by Nathan and co-workers through a chemical-genetic screen. Homologs of MymT are present in all pathogenic mycobacteria. Expression of *mymT* (mt*0196* in CDC1551 or *rv0186A* in H37Rv) is upregulated by multiple transition metals: copper, cadmium, cobalt, nickel and zinc, with the strongest induction by copper. Expression of *mymT* also is increased under conditions mimicking the host macrophage, including nitric oxide and superoxide stress and cell wall disruption. Because *mymT* encodes multiple cysteines organized in typical metal-binding C-X-C or C-X-H motifs, the authors suspected MymT might be a metallothionein. Mass-spectroscopy (MS) analysis showed that MymT binds increasing amounts of copper as the molar ratio of Cu(I) to MymT is increased with the major species containing 4–6 copper ions. Furthermore, degradation of the Cu(I) core of MymT by nitric oxide resulted in the release of copper inside *M. tuberculosis*, suggesting another mechanism for the toxicity of reactive nitrogen species[48]. Importantly, MymT is required for robust growth in excess copper *in vitro*, but is not required for resistance to other divalent metals, indicating that the protein is a copper-specific MT[48]. Additionally, MymT is not required for virulence in mice[48]; this may be due to redundancy in the copper homeostasis mechanisms of *M. tuberculosis* that protect the bacteria even when one copper-handling protein is missing or mutated (Fig. 2A).

The best-characterized bacterial metallothionein is SmtA from *Synechococcus* PCC 7942, which contains a cluster of four zinc ions as well as a zinc-finger domain[65]. Zinc-MTs have been biochemically characterized in *Anabaena* and *Pseudomonas* species but their requirement for zinc tolerance has not been shown[66]. Although a zinc-metallothionein was identified bioinformatically in *E. coli*, biochemical analysis showed the protein bound only a single zinc ion, likely in a zinc-finger domain[66]. It is likely that other bacteria encode MTs, but they may be difficult to identify bioinformatically because of the lack of nucleotide sequence conservation[48, 66], their small size, and by their poorly defined amino acid sequence and structural requirements[61]. Furthermore, bacterial MTs are different from the canonical eukaryotic MTs, in that they can utilize histidine to coordinate metals in the solvent-shielded core[65]. MymT remains a unique example as a prokaryotic copper metallothionein. It will be interesting to see if other bacterial copper MTs can be identified or if this is truly a novel adaptation of *M. tuberculosis* to its host.

The RicR (Rv0190) regulon

Mutations in either the *mpa* (mycobacterium proteasomal ATPase) gene or *pafA* (proteasome accessory factor A), both of which are required for proteasome function, results in the significant repression of expression of almost 40 genes during growth of *M.*

tuberculosis in vitro[49]. A conserved palindromic sequence was found in the putative promoter regions of *lpqS*, *rv2963*, *rv0190*, and *mymT*, as well as two previously unannotated open reading frames, small ORF induced by copper A and B (socAB), suggesting that these genes were regulated by the same transcription factor[49]. Growth experiments confirmed that expression of *lpqS* and *socAB* also are induced by copper[49] as suspected from the previously identified regulation of *rv0190*, *rv2963* and *mymT* by copper[12, 48]. Although the functions of these proteins are unknown except for MymT, it is reasonable to assume that they are also involved in the copper homeostasis mechanisms of *M. tuberculosis*. *LpqS*, *rv2963*, *mymT* and *socAB* are found only in pathogenic mycobacteria, suggesting the proteins encoded by these genes may also be important for virulence.

Rv0190 is similar to *M. tuberculosis* CsoR and has homologs in all mycobacteria and as well as in other bacteria, including *B. subtilis* CsoR[49]. The Cu(I) coordinating amino acids Cys38, His63 and Cys67 (residue numbers based on *M. tuberculosis* CsoR) are conserved in Rv0190 and its homologs[49]. The *lpqS*, *rv2963*, *mymT*, and *socAB* genes are constitutively expressed in the absence of Rv0190[49]. Several other genes, most of which encode hypothetical proteins, were found to be upregulated in the *rv0190* mutant strain compared to wild-type *M. tuberculosis*, including the other genes in the predicted *lpqS* operon[49]. Rv0190 specifically binds to the palindromic sequence in the promoter DNA of at least one of its targets, *lpqS*[49]. The interaction of Rv0190 and the *lpqS* promoter is lost in a dose dependent manner when copper is added[49]. Finally, the *rv0190* mutant is more resistant to copper-stress than wild-type *M. tuberculosis*, likely because the increased expression of genes in the regulon alleviated copper toxicity[49]. Together, the location of the regulatory sequence overlapping with promoters, the increased expression of target genes when Rv0190 is missing, and the loss of promoter-Rv0190 interaction upon addition of copper, indicate that Rv0190 is a copper-responsive repressor (Fig. 2A). Rv0190 was therefore renamed RicR (regulated in copper repressor).

The putative multicopper oxidase Rv0846c

Multicopper oxidases couple the reduction of molecular oxygen to water with the oxidation of a substrate in a copper-dependent manner[67]. Substrates for oxidation include phenolic compounds, amines and other metals, such as iron[67]. MCOs are found throughout eukaryotes, prokaryotes and archaea[68]. Rv0846c of *M. tuberculosis* has amino acid similarity to CueO, a multicopper oxidase of *E. coli*, but has not been characterized yet. Expression of *rv0846c* is upregulated by excess copper[12] and is also upregulated in a *ricR* mutant strain[49], supporting the hypothesis that Rv0846c is involved in copper homeostasis of *M. tuberculosis*. RicR regulates *lpqS* which is located directly downstream of *rv0846c* and, because *rv0846c* is transcribed in the opposite direction of *lpqS*, it is possible that a single RicR binding site regulates expression of both genes (Fig. 2A)[49]. Bioinformatic analysis suggests that Rv0846c is translocated to the periplasm by the Twin-Arginine Translocation (TAT) system and that the amino-terminus is lipidated indicating that, unlike CueO of *E. coli*, Rv0846c may be membrane-associated (Fig. 2AB). The mechanism of regulation, localization, its role in virulence and in detoxifying the periplasm have yet to be determined experimentally for Rv0846c. The fact that *rv0846c* is deleted from a clinical isolate of virulent *M. tuberculosis*[69] indicates that this gene is not required for virulence, probably due to redundant copper detoxification mechanisms.

Copper homeostasis in *E. coli*

The copper homeostasis mechanisms of *E. coli* and *E. hirae* are both well characterized[20, 54]. Because the cell envelope of mycobacteria contains an outer membrane and is more similar to a gram-negative than a gram-positive cell envelope[70], we will use *E. coli* as a comparison to the copper homeostasis mechanisms of mycobacteria. Two primary

copper resistance systems are encoded in the genome of *E. coli*: the inner membrane copper-efflux pump CopA and the multicopper oxidase CueO regulated by CueR, and the CusRS regulated cell envelope-spanning copper-efflux system CusCFBA. Some *E. coli* strains also carry plasmid-encoded copper homeostasis elements, which are reviewed elsewhere[54].

CueR/CopA/CueO

The *cue* (Cu-efflux) regulon comprises the house-keeping copper resistance system of *E. coli*. It is expressed at a basal level at all times and is induced by increasing copper concentrations[15]. CueR (Cu-efflux regulator) is a Cu(I)-sensing transcriptional activator, which binds the *copA* and *cueO* promoters when Cu(I) levels are increased (Fig. 2B)[15, 71]. CueR is a member of the MerR family of transcriptional regulators, many of which respond to heavy metal stress[72]. Promoters regulated by MerR-like proteins are characterized by an elongated spacer between the −10 and −35 promoter elements[72]. MerR-like proteins bind operators between the −10 and −35 regions, and twist the DNA to better align the elements, increasing promoter strength drastically and thus activating transcription[72]. Like other MerR-regulated promoters, the CueR binding site lies between the −10 and −35 elements of its target promoters at *cueO* and *copA*[71].

CueR binds copper and responds to changes in copper concentration with exquisite sensitivity: CueR-mediated induction of *copA* expression is half-maximal at a copper concentration in the zeptomolar range ($2x10^{-21}$ M Cu)[22]. CueR can sense even one “free” copper ion in the cytoplasm and responds to Cu(I), Ag(I) and Au(I) but not to divalent cations such as Zn(II) or Hg(II)[22]. The crystal structure reveals that CueR forms a dimer and each monomer contains a DNA-binding domain, dimerization helix and metal-binding loop (Fig. 2B, PDB 1Q05)[22]. Two cysteines in the metal-binding domain are required for binding of monovalent cations[22]. Although CueR can bind multiple monovalent metal cations, *E. coli* is unlikely to encounter Ag(I) and Au(I) in its natural environments, making the CueR response to Cu(I) the only biologically relevant interaction.

CopA is a metal efflux pump that specifically exports Cu(I) in an ATP-dependent manner (Fig. 2B)[73]. CopA is required for copper resistance of *E. coli in vitro* and during model infection of macrophages[36, 73]. Deletion of *copA* results in intracellular accumulation of copper[74]. Because *copA* is regulated by CueR, its expression is induced by increasing concentrations of copper[15, 71, 73].

CueO (Cu-efflux Oxidase) is a multicopper oxidase required for copper resistance of *E. coli*[75, 76]. Typically multicopper oxidases have at least two copper clusters containing four copper atoms, with additional clusters possible; the type 1 (T1) blue cluster contains one copper atom and is the site of substrate oxidation (or cluster reduction), the combined T2/T3 cluster contains three copper atoms and is the site of oxygen reduction[67, 68]. CueO, like the prototypical multicopper oxidases, contains four copper atoms arranged in the T1 and T2/T3 catalytic sites[75, 77]. A methionine rich helix in CueO binds two additional copper atoms, which increase the catalytic activity of the protein, and a third Cu(I) which is the substrate of oxidation[78]. CueO is likely translocated to the periplasm by the twin arginine protein export system and CueO protects the periplasm from copper-stress (Fig. 2B)[75]. Like other multicopper oxidases, CueO is able to oxidise multiple substrates *in vitro* including Fe(II) and its oxidase activity is copper-dependent[75]. The crystal structure of CueO (PDB 1KV7) confirms that is contains four copper atoms in an organization characteristic of multicopper oxidases[77]. The *in vivo* oxidation substrates of *E. coli* CueO are not known, but it is hypothesized that CueO may oxidize Cu(I) to Cu(II) to protect the periplasm from the more toxic reduced form of copper[15, 75].

Together, the *cue* regulon forms the basis of aerobic copper resistance in *E. coli*. CopA transports excess copper from the cytoplasm to the periplasm and CueO protects the periplasm from toxic copper, probably by oxidizing Cu(I) to the less toxic Cu(II)[77]. The regulator CueR is itself highly effective at preventing free copper in the cytoplasm, based on its extreme affinity for Cu(I)[22].

CusRS and the tripartite efflux pump

The second chromosomally encoded copper response system in *E. coli* is the *cus* (Cu-sensing) locus, and is regulated by a two-component regulatory system: CusR (Cu-sensing regulator) and CusS (Cu-sensing rensor)[79]. CusS senses increases in copper in the periplasm and activates CusR which induces expression of the *cusCFBA* operon, which is divergently transcribed from the *cusRS* operon; CusR likely regulates both promoters (Fig. 2B)[79]. Unlike the *cue* system, *cus* is not expressed at basal levels at all times, it is only induced under extreme copper stress, presumably when the *cue* system is overloaded, and during anaerobic growth[15, 54]. Indeed, during aerobic growth, deletions of *cus* only increase copper sensitivity when *cueO* also is deleted[75]. In a Δ*cueO* mutant, each of the *cusCFBA* genes is required for full copper resistance of *E. coli* during aerobic growth[80].

CusCBA forms a tripartite copper efflux pump and is part of the resistance-nodulation-cell division (RND) family of efflux pumps. RND-type pumps consist of three components, an inner membrane efflux pump, which provides the energy and specificity for the transported substrate, an outer membrane channel and a periplasmic membrane fusion protein which connects both membrane components[81]. It is assumed that the tripartite Cus efflux system exports cytoplasmic copper in one step across both membranes (Fig. 2B). The inner membrane component CusA forms a homotrimer and is likely able to efflux metal ions from both the cytoplasm and periplasm[82]. The movement of ions is dependent on methionine residues lining the channel of the pump[82, 83]. The co-crystal of CusA with the membrane fusion protein CusB shows that six CusB molecules form a complex with three molecules of CusA (PDB 3NE5)[84]. Specifically, two molecules of CusB interact with each other and one molecule of CusA[84]. The crystal structure shows that the trimeric CusC forms a β-barrel in the outer membrane and a barrel composed of α-helices in periplasm(PDB 3PIK)[85]. Together, CusCBA form a complete heavy metal efflux pump, which translocates copper in one step across two membranes out of the cell (Fig. 2B).

The remaining component of the *cus* operon, CusF, is located in the periplasm[80]. CusF binds a single Cu(I) atom, which is likely coordinated by two methionines and one histidine residue[86, 87]. CusF acts as a periplasmic copper chaperone and can directly transfer copper to CusB, thus protecting cells from copper-mediated damage[88–90]. In Fig. 2B we show the Cu(I) bound form of CusF (PDB 2VB2)[87].

The two chromosomally encoded copper-handling systems of *E. coli* have overlapping but distinct functions. CueR regulates the housekeeping copper resistance mechanism, mediated by the inner membrane efflux ATPase, CopA, and the periplasmic multicopper oxidase CueO[15, 54]. Under extreme copper stress or during anaerobic growth, the *cus* system takes on greater importance, removing copper completely from the cell[15, 54].

Conclusions

Several of the *M. tuberculosis* proteins discussed in this review were discovered through experiments initially focused on describing other areas of *M. tuberculosis*'s physiology. The copper sensitive operon, *cso*, was first discovered as part of a genomic island induced during infection[55], MymT was discovered while investigating targets of potential anti-tuberculosis drugs[48], and RicR and it's regulon were found during investigation of the proteasome[49].

These fortuitous discoveries suggest that copper homeostasis mechanisms overlap with other physiological aspects of *M. tuberculosis*.

The variety and increasing numbers of copper-responsive proteins and their mild individual contributions to virulence suggest that there are overlapping mechanisms for copper resistance in *M. tuberculosis*. In *E. coli*, no single protein or complex is entirely responsible for copper resistance, and mutations in multiple systems must be made to confer a strong copper sensitivity phenotype. It has been suggested by several groups that multiple mutations in copper-responsive genes of *M. tuberculosis* must be created in a single strain in order to fully deplete the copper resistance mechanisms and induce a more robust virulence defect[48, 49]. Given the broad effects of copper toxicity, it is also likely that many proteins that are neither copper binding nor copper translocating, may be important in combating copper toxicity; the loss of these seemingly irrelevant proteins could result in copper-sensitivity and virulence defects. By characterizing the many unknown hypothetical proteins and the apparent redundancies in copper resistance, we will develop a much clearer picture of copper homeostasis in *M. tuberculosis* and the role of copper in the host immune response.

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Abbreviations

wt	wild-type
IM	inner membrane
OM	outer membrane

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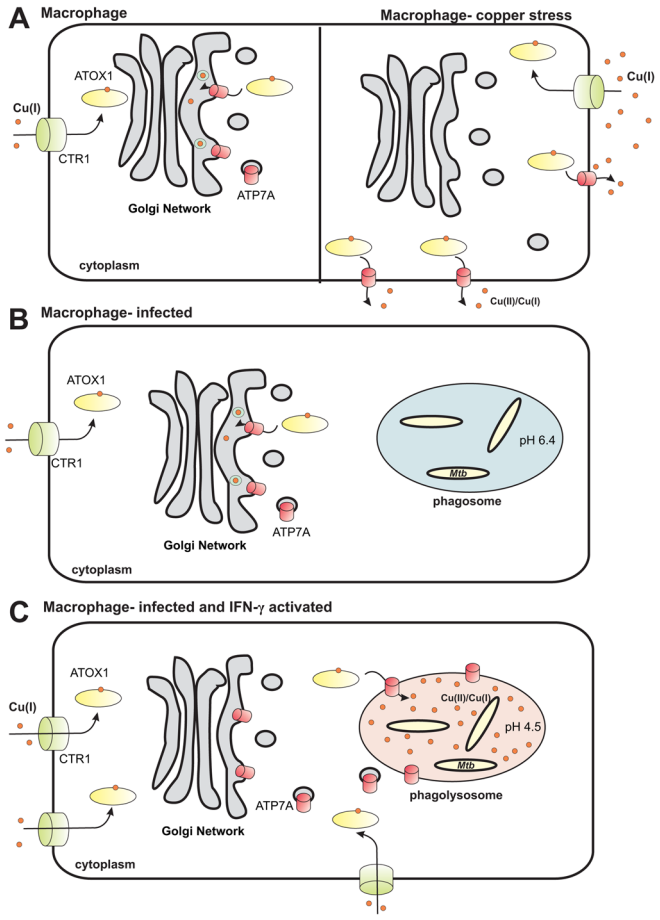

Fig. 1. Copper homeostasis in macrophages

A. In resting macrophages CTR1 imports Cu(I) which is bound by the chaperone ATOX1. ATP7A is localized to the Golgi Network to deliver Cu to copper-requiring proteins. **B.** Under copper-stress ATP7A is trafficked to the membrane to export excess copper, but its transcription and translation are not increased. **C.** Initially upon phagocytosis into macrophages, *M. tuberculosis* suppresses phagolysosome formation. **D.** Once infected macrophages are activated with IFN-γ, phagolysosome fusion proceeds, the pH of the *M. tuberculosis* containing vacuole is reduced to pH4.5, CTR1 and ATP7A expression is increased and ATP7A is trafficked to the phagolysosome.

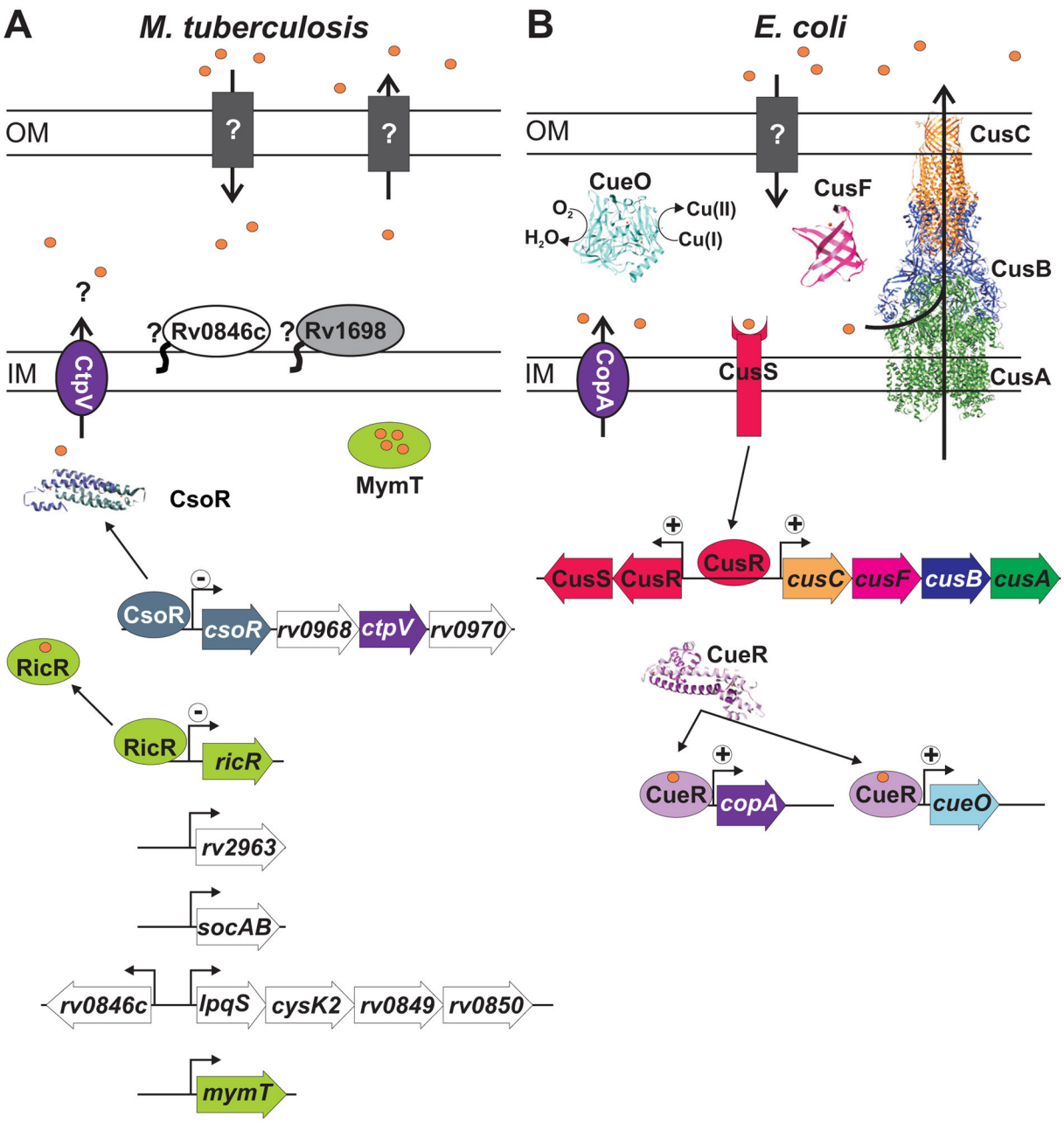

Fig. 2. Copper homeostasis in *M. tuberculosis* and *E. coli*

The thicknesses of the membranes were drawn according to measurements derived from cryo-electron microscopy. The size of the periplasmic space in mycobacteria is larger compared to *E. coli*, but is not shown proportionally in this figure.

A. In *M. tuberculosis* outer membrane proteins involved in copper uptake or efflux are unknown. CtpV is an inner membrane (IM) transporter which likely functions as a copper efflux pump and whose expression is regulated by CsoR (PDB: 2HH7). Rv0846c is a putative multi-copper oxidase. The exact localization of the membrane protein Rv1698 (MctB) which reduces intracellular copper levels is unknown. *MymT* encodes a cytoplasmic copper metallothionein whose expression is regulated by RicR. Other genes in the RicR regulon (*rv2963*, *socAB* and *lpqS*) have undetermined functions. RicR and CsoR are both

repressors which are induced by copper binding. **B.** In *E. coli* the tripartite efflux pump CusABC transports copper from the cytoplasm to the extracellular space (CusAB, PDB: 3NE5; CusC, PDB: 3PIK). CusF (PDB: 2VB2) may act as a periplasmic copper chaperone. CueO (PDB 1KV7) is a multi-copper oxidase and CopA is an IM copper transporting P-type ATPase. The two-component regulator CusSR activates transcription of *cusCFBA*, but does not regulate its own transcription. CueR (PDB 1Q05) binds copper to activate transcription of *copA* and *cueO*. Molecular structures were prepared using the UCSF Chimera program[91].