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Copper Homeostasis in Mycobacteria

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COPPER HOMEOSTASIS IN MYCOBACTERIA

by

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ABSTRACT

Mycobacterium tuberculosis, the causative agent on tuberculosis in humans, is a globally important pathogen. In 2013, nearly 1.2 million people died from tuberculosis. Drug treatments and effective vaccines are lacking for this pathogen. A better understanding of the basic physiology of *M. tuberculosis* is required to improve disease outcomes. Upon inhalation into the lungs, *M. tuberculosis* is taken up by macrophages, in a process called phagocytosis, which normally destroys invading bacteria. As the phagosome inside the macrophage matures, bactericidal mechanisms are activated including: lowering pH, and introducing reactive oxygen and nitrogen species and hydrolytic enzymes to degrade bacterial cell walls. Additionally, macrophages mobilize metals as part of their bactericidal response, by sequestering essential iron and magnesium from bacteria and accumulating toxic levels of copper and zinc. However, *M. tuberculosis* blocks the normal phagosome maturation pathway, and instead survives and replicates inside macrophages.

Based on the observation that copper resistance is required for virulence of *M. tuberculosis* in animal models, we hypothesized that *M. tuberculosis* has multiple copper resistance mechanisms. In order to better understand virulence of *M. tuberculosis*, we aimed to elaborate its copper homeostasis mechanisms. First, we characterized a multicopper oxidase and determined its role in copper resistance. Multicopper oxidases are involved in copper resistance and virulence in some pathogenic bacteria. The *M.*

tuberculosis genome encodes one predicted multicopper oxidase, which is conserved among pathogenic mycobacteria. We showed that this protein indeed has multicopper oxidase activity and that it is required for copper resistance in *M. tuberculosis*. Further, the proteins required for copper entry into mycobacterial cells are unknown. We showed that general porins of *M. smegmatis* and *M. tuberculosis* are required for copper uptake. These results reveal important aspects of copper homeostasis mechanisms in *M. tuberculosis*.

Keywords: *M. tuberculosis*, copper resistance, multicopper oxidase, porin.

Dedication

To my parents.

ACKNOWLEDGEMENTS

I am grateful to my mentor, Michael Niederweis, for constantly challenging me to improve. I am also grateful for the freedom to work on any project that interested me. Not every graduate student can choose her own project and design it from start to finish. More than that, Michael allowed me to pursue projects that were outside his own general interests and even encouraged me along the way. Such freedom has been invaluable in my early career, teaching me how to think about science in both the abstract and concrete. Although this method of progressing is difficult, undoubtedly these skills will prove most useful in my future career. Michael also allowed me to attend many conferences and pursue any opportunities I thought might be useful. Not every mentor supports their students jetting off to South Africa for two months to try something completely new.

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I would not have made it through the long hours, disappointments, and triumphs without my good friends in Birmingham. Rebecca Rudicell supplied listening ears, career advice, and many, many papers. My dear friends Sarah and Adam Sterrett reminded me to think of something other than science every once in a while.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table *Page*

INTRODUCTION

A MULTICOPPER OXIDASE IS REQUIRED FOR COPPER RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

LIST OF FIGURES

Figure *Page*

INTRODUCTION

A MULTICOPPER OXIDASE IS REQUIRED FOR COPPER RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

PORINS INCREASE COPPER SUSCEPTIBILITY OF *MYCOBACTERIUM TUBERCULOSIS*

INTRODUCTION

Mycobacterium tuberculosis as a Global Health Threat

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB) in humans, is a globally important pathogen. It is estimated that one-third of the world's population is infected with *M. tuberculosis*; in 2012 there were 8.6 million new infections and 1.2 million deaths in 2013 (WorldHealthOrganization, 2013). Currently there is one vaccine against *M. tuberculosis* infection, the live, attenuated strain *M. bovis* bacillus Calmette-Guerin (commonly called the BCG vaccine) with limited efficacy, protecting zero to eighty percent of recipients depending on a variety of factors (Brandt *et al.*, 2002). Immunity from the BCG vaccine wanes early, limiting its protection to children under 10 years of age (Brandt *et al.*, 2002). Without a vaccine offering strong, life-long protection in a greater portion of the population, antibiotics are required to treat tuberculosis and limit disease spread.

Drug treatment regimens against *M. tuberculosis* require multiple antibiotics given over a period of at least six months, sometimes extending up to two years. The length, difficulty and cost of treatment can lead to poor compliance among patients, resulting in the development of drug resistant strains. Additionally, the prevalence of multi- and extensively-drug resistant strains is increasing (WorldHealthOrganization, 2013). While cure rates for drug-susceptible TB are high (up to 95% if the regimen is completed as directed) (Koul *et al.*, 2011), the emergence and spread of drug resistant TB portends a global health crisis for which new treatments and better vaccines must be

found. In order to develop these new tools, a better understanding of the pathogen and its interactions with the host is needed.

M. tuberculosis and the Host

M. tuberculosis is an intracellular pathogen that survives and replicates inside host alveolar macrophages. Normally, macrophages that encounter invading bacteria undergo a regulated process to kill and destroy the bacteria. Macrophages first engulf the bacterium by phagocytosis and the phagosome matures through a series of fusions with other compartments. This maturation process results in a toxic, bactericidal environment including: reactive oxygen species (ROS) and reactive nitrogen species (RNS) which damagae proteins, DNA and lipids; and lipases, proteases and hydrolases to degrade the cell wall (Figure 1) (Flannagan *et al.*, 2009). Additionally the mature phagosome is a nutrient poor environment (Flannagan *et al.*, 2009). *M. tuberculosis* inhibits the normal phagosomal maturation process at some point along the continuum, thereby allowing survival and growth (Flannagan *et al.*, 2009). However, upon interferon-γ activation, macrophages are able to overcome this block and increase their bactericidal activity against *M. tuberculosis* (MacMicking *et al.*, 2003; Schaible *et al.*, 1998). Therefore, *M. tuberculosis* has developed mechanisms to resist the stressful environment of the macrophage phagosome; chief among these is the highly impermeable outer membrane (Niederweis *et al.*, 2010; Purdy *et al.*, 2009).

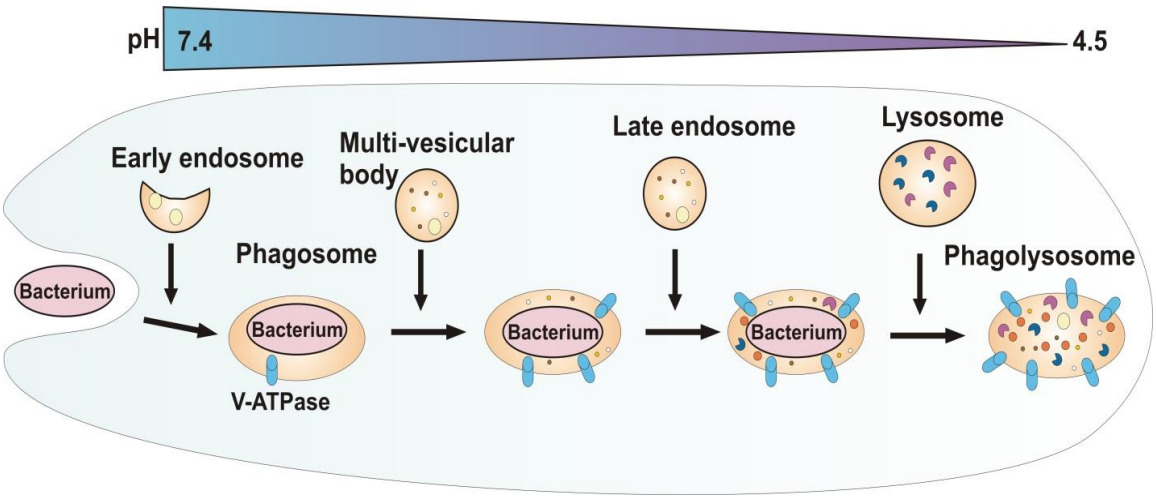

Figure 1. Macrophage phagosome maturation. Upon encountering an invading bacterium, macrophages undergo a regulated process to take up and destroy the bacterium. As the phagosome matures, stresses are introduced and increased. V-ATPases pump protons into the environment, reducing the pH. Reactive oxygen species and reactive nitrogen species are created to increase the bactericidal activity of the environment. Proteases, lipases and hydrolases are introduced to degrade the bacterial cell wall (Flannagan *et al.*, 2009).

Metals and the Bactericidal Activity of Macrophages

In addition to introducing chemical and proteinaceous stressors to the phagosome, macrophages also mobilize essential metals to increase bactericidal activities (Figure 2). Many metals are required for life: iron and copper are used in electron transport and energy generation; manganese, magnesium and calcium are required for transcription and translation; zinc is required in enzymes and in regulatory proteins. In the prototypical example of withholding an essential metal, iron is excluded from the phagosome. NRAMP1 cation transporters pump iron and manganese out of the phagosome and sequestered by other proteins: ferritin binds iron intracellularly and, lactoferrin and transferrin bind iron in extracellular spaces; calprotectin binds manganese extracellularly (Figure 2) (Hood and Skaar, 2012). Thus, *M. tuberculosis* employs mechanisms to obtain

necessary levels of iron by deploying small molecule iron chelators, called siderophores (Rodriguez, 2006).

In contrast, essential metals that are also highly toxic such as copper and zinc, are actively pumped into the phagosome and increase its bactericidal activity (Figure 2). Zinc likely exerts bactericidal activity through metal ion replacement in proteins and enzymes that require other metals (i.e. manganese) for function (McDevitt *et al.*, 2011). Copper is toxic through a variety of mechanisms, due to its high redox potential (Crichton and Pierre, 2001). The mechanisms of copper toxicity and its role in controlling infections will be discussed in greater detail below.

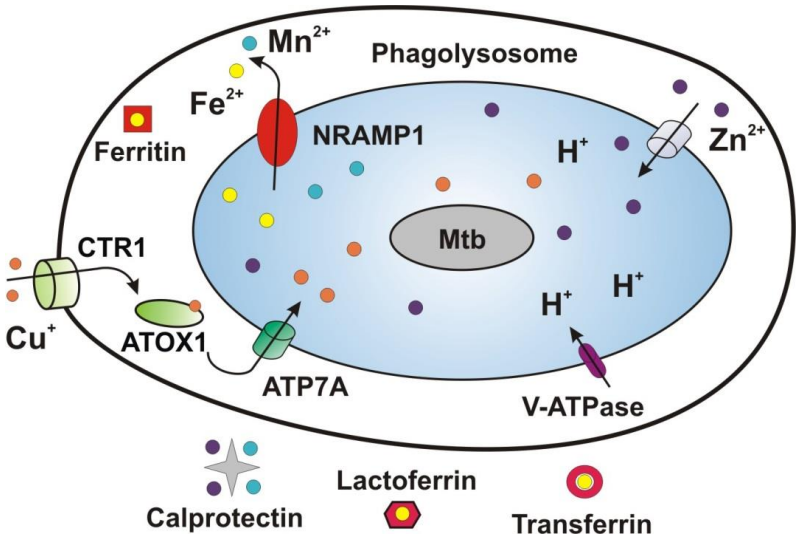

Figure 2. Mobilization of metals in macrophages in response to infection. V-ATPases pump protons into the phagosome to reduce pH. The cation transporter NRAMP1 effluxes iron (Fe^{2+}) and manganese (Mn^{2+}) out of the phagosome, starving the bacterium (Mtb) of these essential metals (Hood and Skaar, 2012). An unknown zinc transporter pumps Zn^{2+} ions into the phagosome, increasing stress on the bacterium through zinc toxicity (Botella *et al.*, 2011). Increased CTR1 pumps copper ions into the cytoplasm where they are immediately bound by the chaperone ATOX1. ATP7A abundance increases and the protein relocalizes from the golgi network to the phagosomal membrane, resulting in increased toxic copper in the phagosome (White *et al.*, 2009).

The Dual Nature of Copper

Copper is essential in almost all organisms due to its redox potential (Pena *et al.*, 1999); copper is readily able to cycle between the Cu^{1+} and Cu^{2+} states in biological systems (Crichton and Pierre, 2001). Copper is an important cofactor in a variety of enzymes such as: tyrosinases, used in melanin generation; amine oxidases, which are involved in neurotransmitter generation; methane monooxygenases, used in energy generation by methanotrophs; multicopper oxidases, which oxidize a wide variety of substrates; and superoxide dismutases, to protect cells against oxidative stress (Balasubramanian and Rosenzweig, 2007; Grass *et al.*, 2011; Linder and Hazegh-Azam, 1996; Pena *et al.*, 1999). In *M. tuberculosis*, there are at least two enzymes known to require copper for activity. Copper is required as the terminal electron acceptor in cytochrome c oxidase, an enzyme essential for aerobic growth (Shi *et al.*, 2005). The Cu,Zn superoxide dismutase of *M. tuberculosis*, SodC, is required for virulence to combat the oxidative burst within host macrophages (Piddington *et al.*, 2001). The role of copper in energy generation and oxidative radical reduction make this element essential for cellular viability.

However, copper is also toxic to cells in high concentrations. Copper exerts toxicity through a variety of methods. *In vitro* copper is able to produce hydroxyl radicals from hydrogen peroxide through the Fenton reaction (reactions 1-2 below), but this is unlikely to occur in the cytoplasm of bacterial cells (Macomber *et al.*, 2007).

$$(1) \qquad X_{red} + Cu^{2+} \rightarrow X_{ox} + Cu^{1+}$$

$$(2) \qquad Cu^{1+} + H_2O_2 \rightarrow Cu^{2+} + OH^- + HO^\cdot$$

Bacteria strongly regulate the availability of copper, by sequestration and efflux; indeed, there is likely no free copper in the cytoplasm of bacterial cells under standard conditions (Changela *et al.*, 2003; Outten and O'Halloran, 2001). Furthermore, copper appears more toxic under anaerobic conditions, when Fenton chemistry is impossible (Macomber and Imlay, 2009). Instead of acting by Fenton chemistry, copper is toxic through other means. Copper displaces iron-sulfur clusters and causes their breakdown, inactivating a number of enzymes and other proteins (Macomber and Imlay, 2009). Copper can also induce incorrect disulfide bond formation, inactivating proteins in the periplasm (Hiniker *et al.*, 2005). Finally, copper can oxidize lipids, inducing membrane damage (Dupont *et al.*, 2011). Cells must maintain the proper balance of copper required for function while limiting the damage from this highly redox active metal.

Copper is Required by Mammalian Immune Systems

Copper is required for the proper growth and development of animals on a total organismal level. Complete loss of the copper transporter ATP7A results in embryonic lethality, likely due to loss of activity of several copper-containing enzymes, which are required for embryogenesis (Wang *et al.*, 2012). For example, among other defects, *atp7a* null embryos fail to develop proper vasculature. Lysyl oxidase is an enzyme involved in connective tissue generation and proper development of vasculature; it is likely this enzyme has reduced copper occupancy and thus reduced function in the mice lacking ATP7A (Wang *et al.*, 2012).

Copper is also required for the development of the human immune system, through a variety of mechanisms. Copper plays an important role in the generation of

antibodies, and is therefore a critical component of the adaptive immune response (Prohaska and Lukasewycz, 1981). Humans and animals living on a copper deficient diet show a variety of changes in immune effector cells and functions. Copper deficiency causes atrophy and loss of function of the thymus and reduced T-cell proliferation, decreased numbers of circulating monocytes and neutrophils, and decreased cytokine secretion (Munoz *et al.*, 2007). Furthermore, copper deficiency is associated with reduced respiratory burst and phagocytic activity of macrophages (Munoz *et al.*, 2007).

In humans, individuals with the hereditary Menkes disease, the result of mutations in the copper transporter ATP7A, are more susceptible to a variety of bacterial and fungal infections (Failla and Hopkins, 1998). Children surviving on nutrient-limited diets are also more susceptible to infection, which can be alleviated with the simple addition of extra copper in their diets (Castillo-Duran *et al.*, 1983). These afflictions highlight the importance of copper to promote a fully functional immune system.

Importantly, copper plays a direct role in the innate immune response. Addition of copper to growth medium increases the bactericidal activity of macrophages (White *et al.*, 2009). Macrophages actively accumulate copper in bacteria containing phagosomes (Wagner *et al.*, 2005). Copper transporters CTR1 and ATP7A are responsible for the increase in cellular copper and phagosomal copper, respectively (Figure 2). Expression of both *atp7a* and *ctr1* is increased in macrophages upon exposure to lipopolysaccharide or interferon-γ activation (White *et al.*, 2009). Upon phagocytosis of a bacterium, ATP7A relocalizes from the golgi network to the phagosome (Figure 2), where the copper concentration increases (Wagner *et al.*, 2005; White *et al.*, 2009). Bacteria require copper resistance mechanisms in order to survive the increased copper in the phagosome (White

et al., 2009). Furthermore, when ATP7A protein levels are reduced, bacteria are better able to survive in the phagosome (White *et al.*, 2009). The molecular involvement of ATP7A in controlling infections partially explains why children with Menkes disease suffer a higher rate of bacterial infections.

Copper Resistance is Required for Virulence

Copper resistance is required for virulence of both Gram-negative and Gram-positive species of bacteria. In particular, several intracellular pathogens require copper efflux for survival in macrophages. The intracellular pathogen *Salmonella enterica sv.* Typhimurium (*S.* Typhimurium) requires copper effluxing P-type ATPases for survival in macrophages (Osman *et al.*, 2010). The Gram-positive *Listeria monocytogenes*, another intracellular pathogen, also requires copper efflux for persistence in the liver in a mouse infection model (Francis and Thomas, 1997). CopA1 and CopA2, the copper efflux pumps of *Pseudomonas aeruginosa*, an extracellular pathogen, are required to establish infection in an *Arabidopsis thaliana* model system (Gonzalez-Guerrero *et al.*, 2010).

Copper efflux is not the only mechanisms of copper resistance required for virulence. The multicopper oxidase CueO of *S.* Typhimurium is required for full mouse colonization, but not for resistance to killing by macrophages (Achard *et al.*, 2010). A periplasmic copper chaperone of *Streptococcus pneumoniae* is required for copper resistance and virulence (Fu *et al.*, 2013; Shafeeq *et al.*, 2011).

New evidence suggests that even siderophores, the keystone of iron acquisition during virulence, play an active role in protecting pathogenic bacteria against copper-mediated killing. Yersiniabactin, a siderophore of uropathogenic *E. coli*, binds copper

and protects against copper toxicity both extracellulary and upon phagocytosis by macrophages (Chaturvedi *et al.*, 2012; Chaturvedi *et al.*, 2013). Thus, many mechanisms of copper resistance contribute to virulence or survival of bacteria.

Copper Homeostasis Mechanisms of *E. coli*

The copper resistance mechanisms of several model bacteria, including the Gram-negative *E. coli* and Gram-positive *Enterococcus hirae*, are well described (Rensing and Grass, 2003; Solioz and Stoyanov, 2003). In contrast, those of *M. tuberculosis* are relatively obscure. Because *M. tuberculosis* has an outer membrane (Hoffmann *et al.*, 2008), it is useful to compare to *E. coli* and other Gram-negative species to identify potential components of copper resistance (Figure 3).

It is unknown how copper enters Gram-negative cells across the outer membrane. It is assumed that general porins are the main entry point for copper into cells. Mutations in *E. coli* that resulted in copper resistance pointed to outer membrane proteins for copper entry (Lutkenhaus, 1977), but this research was not confirmed (Li *et al.*, 1997). Paradoxically, deletion mutants of several genes encoding general porins resulted in increased copper susceptibility in *E. coli* (Egler *et al.*, 2005). The methanotrophic bacterium *Methylosinus trichosporium* OB3b utilizes two pathways to obtain copper, likely because of its high copper requirements (Balasubramanian *et al.*, 2011). First, *M. trichosporium* utilizes copper-binding methanobactin to bind and retrieve copper from its environment, in a system analogous to siderophore-mediated iron uptake (Balasubramanian *et al.*, 2011). Secondly, *M. trichosporium* utilizes general porins to take up additional copper, although the specific identities of such porins are unknown

(Balasubramanian *et al.*, 2011). Thus the mechanism for copper entry in many bacteria remains enigmatic.

E. coli uses several overlapping mechanisms to respond to copper overload and prevent toxicity. There are two chromosomally encoded copper efflux systems: a P-type ATPase, CopA, pumps copper from the cytoplasm to the periplasm while the CusABC tripartite efflux pump moves copper from the cytoplasm and periplasm to the extracellular space (Rensing and Grass, 2003). An additional component of the Cus system, CusF, acts as a periplasmic copper chaperone and may deliver copper to CusABC (Bagai *et al.*, 2008). A multicopper oxidase, CueO, prevents copper toxicity in the periplasm, likely by oxidizing toxic Cu^{1+} to less toxic Cu^{2+} (Grass and Rensing, 2001; Singh *et al.*, 2004). The transcriptional regulator CueR is an activator that increases transcription of *copA* and *cueO* when cytoplasmic copper levels increase (Outten *et al.*, 2000; Stoyanov *et al.*, 2001). The two-component regulator CusRS increases expression of the *cusCFBA* operon when periplasmic copper increases (Outten *et al.*, 2001).

Copper Homeostasis Mechanisms of *M. tuberculosis*

Despite being studied since 1882 (Koch, 1882), much of the basic physiology of *M. tuberculosis* is poorly understood. The full complement of copper resistance mechanisms in *M. tuberculosis* has not been characterized (Rowland and Niederweis, 2012). In-depth characterization of copper homeostasis in *M. tuberculosis* will provide valuable insight into the ability of the bacterium to survive and replicate in the toxic macrophage phagosome.

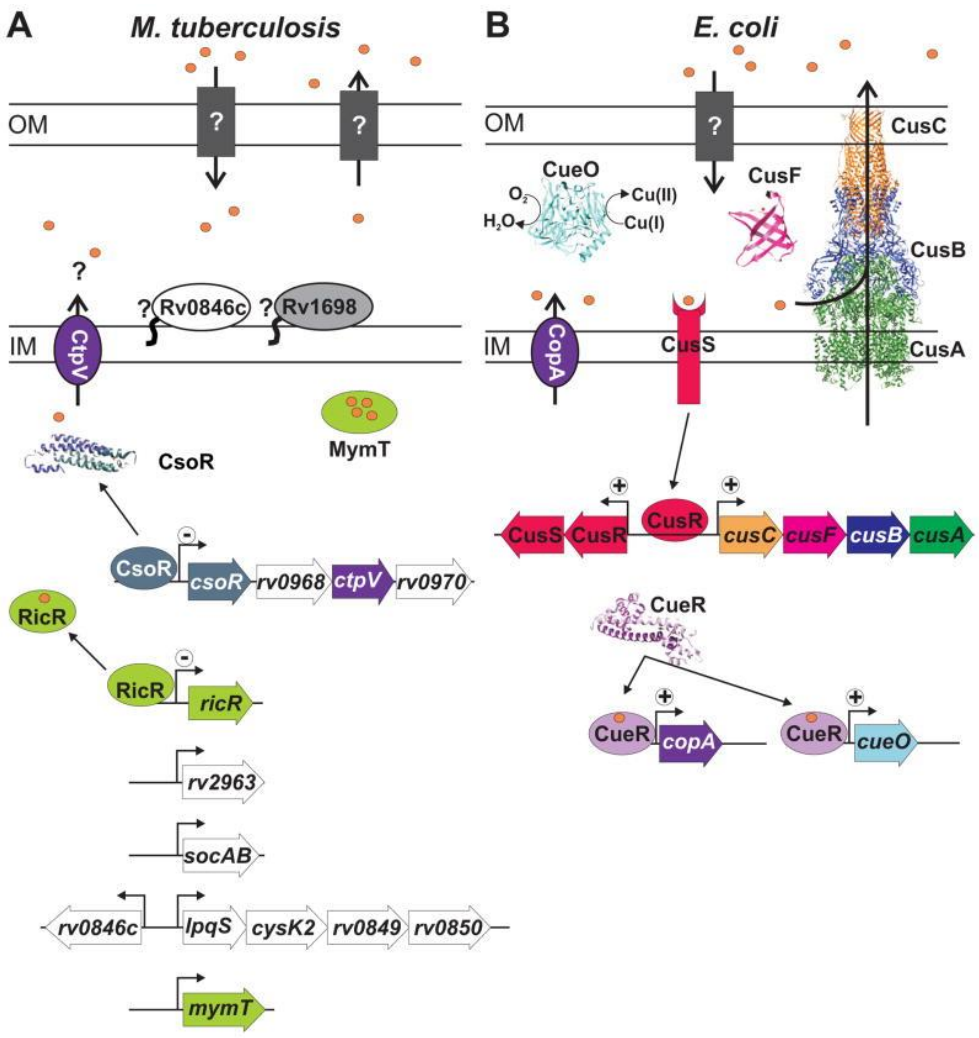

Figure 3. Copper homeostasis in *M. tuberculosis* and *E. coli*. The thicknesses of the membranes were drawn according to measurements derived from cryo-electron microscopy. The size of the periplasmic space in mycobacteria is larger compared to *E. coli*, but is not shown proportionally in this figure. 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 (Pettersen *et al.*, 2004). Figure and legend reused with permission (Rowland and Niederweis, 2012).

There are many differences in the copper homeostasis machinery of *M. tuberculosis* compared to *E. coli* (Figure 3). While there are known general porins in *M. smegmatis*, no such proteins exist in *M. tuberculosis*. It is unknown how copper is transported across the outer membrane of *M. tuberculosis*. Like *E. coli*, *M. tuberculosis* has a P-type ATPase, CtpV, that effluxes excess copper from the cytoplasm to the periplasm; CtpV partially contributes to virulence of *M. tuberculosis* (Ward *et al.*, 2010). There is no known tripartite copper efflux system in *M. tuberculosis* and no homologues of any of the proteins in the CusABCF system. *M. tuberculosis* has an additional component to protect against copper stress, copper metallothionein MymT, which binds four to six copper ions, sequestering them from the cytoplasm (Gold *et al.*, 2008). Copper metallothioneins are common in eukaryotes, but as yet there is no data that they are widely distributed in prokaryotes (Blindauer, 2011). While MymT has high copper binding capacity and contributes to *in vitro* copper resistance, it is not required for virulence (Gold *et al.*, 2008).

Another component of copper resistance in *M. tuberculosis* is the MctB/Rv1698 protein, encoded by gene *rv1698*. MctB is conserved among mycolic acid containing bacteria (i.e. all mycobacteria and corynebacteria) but has no predicted function (Siroy *et al.*, 2008). MctB is required for copper resistance and virulence in *M. tuberculosis*

(Wolschendorf *et al.*, 2011). Initially, MctB was thought to be an outer membrane component of copper efflux, however, more recent evidence revealed that MctB is localized to the periplasm and is not surface accessible (Figure 3) (Siroy, *et al.* in preparation). MctB is membrane associated through a hydrophobic N-terminal helix, which remains uncleaved after translocation to the periplasm (Figure 3) (Siroy, *et al.*, in preparation). Thus, MctB contributes to copper resistance of *M. tuberculosis* through an unknown mechanism.

There are two known copper responsive transcriptional regulators in *M. tuberculosis* (Figure 3). The regulator CsoR binds excess copper in the cytoplasm, releasing its target DNA and allowing transcription to proceed (Liu *et al.*, 2007). CsoR regulates only its own operon, which includes *ctpV* (*rv0969*) and two other genes encoding proteins with unknown functions (Rv0968 and Rv0970) (Liu *et al.*, 2007). In contrast, RicR is a global copper responsive regulator. RicR acts in a manner similar to CsoR, by binding copper and derepressing transcription (Festa *et al.*, 2011). RicR regulates expression of the metallothionein *mymT* and many genes whose protein products have unknown functions (Festa *et al.*, 2011). RicR also regulates expression of *rv0846c*, a putative multicopper oxidase (Festa *et al.*, 2011). Rv0846c is conserved among pathogenic mycobacteria, but notably lacking from non-pathogenic species, indicating a potential role in virulence (Table 1).

Table 1. Conservation of Rv0846c putative multicopper oxidase amongst mycobacteria by percent amino acid identity.

Organism	Protein	Identity
M. tuberculosis	Rv0846c	100
M. bovis	Mb0869c	99
M. marinum	MMAR_4770	71
M. avium avium	MaviaA2_03747	70
M. avium paratuberculosis	MAP0701c	69
Corynebacterium glutamicum	Cg3287 (NCgl2865)	43
C. diphtheriae	CDHC02_0060	42
E. coli	CueO	23

Aims of this Dissertation

In this work the components of copper homeostasis in *M. tuberculosis* are further characterized. Because multicopper oxidases play an important role in copper resistance and virulence in other bacteria, we investigated the putative multicopper oxidase of *M. tuberculosis*, Rv0846c. First we showed the Rv0846c has bona fide multicopper oxidase activity. Second we characterized the role of Rv0846c in copper resistance of *M. tuberculosis*. Additionally, we sought to further elucidate the pathways of copper entry into *M. tuberculosis*. As copper accumulation is toxic to *M. tuberculosis* (Gold *et al.*, 2008; Ward *et al.*, 2010; Wolschendorf *et al.*, 2011), understanding the mechanisms of cell entry may reveal new ways to exploit natural uptake systems. We characterized the role of general porins in copper uptake and susceptibility in the model organism *M. smegmatis*. Further, we showed that as yet unidentified pores were required for copper susceptibility in *M. tuberculosis.*

A MULTICOPPER OXIDASE IS REQUIRED FOR COPPER RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

by

JENNIFER L. ROWLAND AND MICHAEL NIEDERWEIS

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ABSTRACT

Mycobacterium tuberculosis, the causative agent of tuberculosis, is one of the most important bacterial pathogens. Recent work has revealed that the natural bactericidal properties of copper are utilized by the host immune system to combat infections with bacteria including *M. tuberculosis*. However, *M. tuberculosis* employs multiple mechanisms to reduce the internal copper amount by efflux and sequestration, which are required for virulence of *M. tuberculosis*. Here, we describe an alternative mechanism of copper resistance by *M. tuberculosis*. Deletion of the *rv0846c* gene increased the susceptibility of *M. tuberculosis* to copper by at least ten-fold, establishing Rv0846c as a major component of copper resistance in *M. tuberculosis*. *In vitro* assays showed that Rv0846c oxidized organic substrates and Fe(II). Importantly, mutation of the predicted copper coordinating cysteine 486 resulted in inactive Rv0846c protein which did not protect *M. tuberculosis* against copper stress. Hence, Rv0846c is a multicopper oxidase of *M. tuberculosis* and was renamed mycobacterial multicopper oxidase (MmcO). MmcO is membrane associated, probably by lipidation after export across the inner membrane by the Twin-Arginine Translocation system. However, mutation of the lipidation site did not affect the oxidase activity or the copper protective function of MmcO. Our study revealed MmcO as an important copper resistance mechanism of *M. tuberculosis*, which possibly acts by oxidation of toxic Cu(I) in the periplasm.

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis, kills over one million people each year and the global occurrence of multi-drug and extensively drug resistant strains is increasing (1). Understanding the survival mechanisms of *M. tuberculosis* in the human host is key to controlling this devastating disease. In addition to the known mechanisms of the innate and adaptive immune response to infections (2, 3), it is increasingly recognized that copper is utilized by macrophages to kill pathogenic bacteria (4, 5). In most organisms copper is used as a cofactor in a variety of enzymes including cytochrome c oxidases and is therefore an essential micronutrient (6). However, copper is also toxic by a variety of mechanisms: lipid peroxidation (7), metal ion replacement in proteins (8), formation of spurious disulfide bonds (9), and oxidation and degradation of iron-sulfur clusters in proteins (10). Hence, cells employ defense mechanisms against copper poisoning while maintaining sufficient intracellular copper levels (11, 12).

Copper also is utilized in host immune systems to prevent infection (reviewed in (13)). Not only is copper required for proper development of the immune system (14), new evidence shows that copper is employed at a cellular level to kill invading bacteria. Macrophages increase intracellular copper concentrations in response to multiple bacteria including *M. tuberculosis* (5, 15, 16). Additionally we have shown that copper accumulates in granulomas of guinea pigs infected with *M. tuberculosis*, and that copper resistance is required for full virulence in *M. tuberculosis* (4).

The mechanisms of copper homeostasis in mycobacteria include copper efflux and sequestration of cytoplasmic copper by the metallothionin MymT (13, 17-19).

Multicopper oxidases play a crucial role in copper detoxification in many bacteria including *E. coli* (20), *Pseudomonas syringae* (21), *Salmonella enterica* (22) and others (23, 24). Multicopper oxidases are also required for virulence in *Salmonella* (22) and *Xanthomonas campestris* (25). However, it is unknown whether the putative multicopper oxidase Rv0846c plays a role in copper resistance in *M. tuberculosis*. Multicopper oxidases catalyze the single electron oxidation of four substrate equivalents coupled with the reduction of oxygen to water (26). Four copper ions coordinated in two centers comprise the multicopper oxidase active site (27). The Type 1 copper center is the site of substrate oxidation and the Cu coordination by cysteine gives multicopper oxidases their characteristic blue color (27). The coupled Type 2 and Type 3 copper centers are the site of oxygen reduction (27). Multicopper oxidases are conserved throughout all kingdoms of life and prominent examples include fungal laccases, and human ceruloplasmin (26, 27). Multicopper oxidases can oxidize a variety of substrates including phenolic compounds such as siderophores and lignins (26).

Here we show that Rv0846c is a *bona fide* multicopper oxidase capable of oxidizing known organic substrates and Fe(II). Thus, we have renamed Rv0846c as MmcO for mycobacterial multicopper oxidase. Importantly, the susceptibility of the *ΔmmcO* mutant to copper *in vitro* was increased by more than 10-fold compared to wild-type *M. tuberculosis*, indicating that MmcO also oxidizes toxic Cu(I). We also showed that the conserved cysteine 486 in the active site is required for oxidase activity and copper resistance. MmcO is membrane associated, possibly through a lipidation site at cysteine 35. Together, these results demonstrate that MmcO plays an important role in copper resistance in *M. tuberculosis*.

MATERIALS AND METHODS

Chemicals, Enzymes, and DNA

Hygromycin B was purchased from Calbiochem. All other chemicals were purchased from Merck, Roche or Sigma at the highest purity available. Enzymes for DNA restriction and modification were purchased from New England Biolabs. Isolation and modification of DNA was performed using standard protocols (28). Oligonucleotides were obtained from Integrated DNA Technologies (Table 2).

Bacterial Strains, Media, and Culture Conditions

Bacterial strains used in this work are described in Table 1. *E. coli* was grown routinely in LB medium at 37°C with shaking. *M. tuberculosis* H37Rv cultures and its derivatives were grown in Middlebrook 7H9 liquid medium supplemented with 0.2% glycerol, OADC (8.5 g/L NaCl, 20 g/L dextrose, 50 g/L bovine albumin (fraction V), 0.03 g/L catalase, 0.6 ml/L oleic acid), 0.02% Tyloxapol, or on Middlebrook 7H10 agar supplemented with 0.5% glycerol using premixed powders (Difco). The avirulent *M. tuberculosis* strain $mc^2$6230 (kind gift from Dr. Jacobs) and its derivatives were grown in the same media as H37Rv with the addition of 24 μg/mL pantothenate and 0.2% casamino acids (acid hydrolyzed). Copper was added when required in the form of $CuSO_4$ at various concentrations. Antibiotics were used at the following concentrations when required: hygromycin, 200 μg/mL for *E. coli* or 50 μg/mL for mycobacterial strains; kanamycin, 30 μg/mL.

Plasmid Construction

E. coli strain DH5α was routinely used for plasmid construction and propagation. Plasmids used in this work are described in Table 1; primers used are described in Table 2. Plasmid pML1641 was generated by PCR amplifying *mmcO* from the wt *M. tuberculosis* chromosome using primers CN1695 and CN1698, the product was digested with SphI, primer CN1698 added a SwaI half-site, and ligated into pMN016(29) which was digested with SphI and SwaI. Plasmid pML1648 was generated by amplifying the upstream homologous region of *mmcO* using primers CN1702 and CN1703 which added PacI and NsiI restriction sites respectively; the PCR product and empty knockout vector pML523 (30) were digested with PacI and NsiI and ligated. The downstream homologous region of *mmcO* was amplified with primers CN3119 and CN1700 which added an SpeI and SwaI half-site respectively; the PCR product was digested with SpeI and the vector pML1648 was digested with SpeI and SwaI and the products were ligated together to generate pML1649, the final *mmcO* knockout vector. The mmcO knockout vector was designed such that 9 bp upstream of the *mmcO* start codon and the entire *mmcO* gene, except the last 13 bp, were deleted. Plasmid pML1227 was generated by amplifying *mmcO* from pML1641 using primers CN1695 which added an SphI site and ribosome binding site, and CN3120 which added the Strep-Tag (II) and HindIII restriction site; the PCR product and backbone vector pMN016 were digested with SphI and HindIII and ligated together. Residue Cys35 was mutated to Ala by CCR (31) using primers CN145, CN110 and CN2863 (the mutagenesis primer); product was generated by PCR amplification from pML1641, digested with SphI and HindIII and ligated into pMN016 digested with the same enzymes, resulting in pML1252. Residue Cys486 was mutated to

Ala by CCR (31) using primers CN145, CN110 and CN2873 (the mutagenesis primer); the sequence was amplified from pML1641. The product was digested with SphI and HindIII and ligated into pMN016 digested with the same enzymes, resulting in plasmid pML1262. Plasmid pML1231 was generated by amplifying *mmcO* without TAT secretion signal (amino acid residues 2-35, abbreviated $mmcO_{\Delta 2\text{-}35}$) from pML1227 with primers CN2484 and CN2398 which added NdeI and HindIII respectively; the PCR product and vector pET21(a+) were digested with NdeI and HindIII and ligated together.

Table 1. Strains and plasmids used in this work.

Strain	**Parent strain and relevant genotype**	**Source or reference**
E. coli DH5α	*recA1; endA1; gyrA96; thi; relA1; hsdR17(rK-;mK+); supE44; f80ΔlacZΔM15;* D*lacZYA-argF; UE169*	Sambrook, *et al.* (28)
E. coli BL21(DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3*	Novagen
M. tuberculosis H37Rv	wild-type	ATCC# 25618
M. tuberculosis $mc^2$6230	H37Rv Δ*RD1* Δ*panCD*	Dr. Jacobs(32)
M. tuberculosis ML1221	$mc^2$6230 Δ*mmcO*::pML1649(loxP-gfp_m^{2+}-*hyg*-loxP)	this study
M. tuberculosis ML1222	$mc^2$6230 Δ*mmcO*::loxP	this study
M. tuberculosis ML413	$mc^2$6230 Δ*rv1698*::loxP	this study
M. tuberculosis ML1223	H37Rv Δ*mmcO*::pML1649(loxP-gfp_m^{2+}-hyg-loxP)	this study
M. tuberculosis ML1224	H37Rv Δ*mmcO*::loxP	this study
Plasmid	**Parent vector, relevant genotype and properties**	**Source or reference**
pET-21(a+)	T7 promoter, transcription start and terminator, His-tag, *lacI*, *aph*, pBR322 ORI; 5443 bp	Novagen, WI
pCreSacB1	p_{groEL}-*cre*, oriE, oriM, *sacR*, *sacB*, *aph*; 7891 bp	Dr. Steyn

pMS2	ColE1 origin, *hyg*, oriM; 5229 bp	(33)
pMN016	ColE1 origin, *hyg*, oriM, p_{smyc}-*mspA*; 6164 bp	(29)
pML523	pUC origin, pAL5000ts origin; *sacB xylE* loxP-gfp_m^{2+}-*hyg*-loxP; 9845 bp	(30)
pML1648	pML523; $mmcO_{up}$-loxP-gfp_m^{2+}-*hyg*-loxP; 10875 bp	This study
pML1649	pML1648; loxP-gfp_m^{2+}-*hyg*-loxP-$mmcO_{down}$; 11857 bp	This study
pML1227	pMN016; *mmcO*-Strep-Tag II; 7055bp	This study
pML1641	pMN016; *mmcO;* 7027 bp	This study
pML1252	pMN016; $mmcO_{C35A}$*;* 7027 bp	This study
pML1262	pMN016; $mmcO_{C486A}$; 7027 bp	This study
pML1231	pET-21(a+); $mmcO_{\Delta 2\text{-}35}$- Strep-Tag II; 6836 bp	This study

[a]Gfp_m^{2+} refers to a mutant Gfp with enhanced folding and fluorescence. The codon usage for gfp_m^{2+} was adapted for expression in mycobacteria as described by Steinhauer, *et al.*(75). p_{smyc} refers to the mycobacterial promoter smyc. Subscripts "up" and "down" refer to upstream and downstream homologous regions used for recombination and knockout generation.

Table 2. Oligonucleotides used in this work.

Oligonucleotide	Sequence (5'→3')
CN110	CGTTCTCGGCTCGATGATCC
CN145	CGACCAGCACGGCATACATC
CN1695	AAGCATGCGCAGAAAggaggttaatATGCCCGAGCTGGCCACG AG (sphI)
CN1698	AAATGTCACAGAATGTAGTCCAG (swaI half site)
CN1700	AAATCTCGTCTGGATTTGGTCTCGCTC (swaI half site)
CN1702	ACGAGGTTAATTAACTACATTCTGTGACAGGCGGCTTG (pacI)
CN1703	CATATGCATGGTGCTGGCCTGTACGCTAG (nsiI)
CN2398	CATGGTTCGAAAAGCTTATTTAAATCTACTTCTCGAACTG CGGG (hindIII)
CN2484	GGAGATATACATATGGCCTCGAAGCCCACGGCATCCGGC GCC (ndeI)
CN2863	GCTTTGCGCTAGCCGCT***GCT***GCCTCGAAGC
CN2873	GTGTGGGTGATGCAC***GCC***CACAACAACTATCAC
CN3119	ACTAGTCACCTTACCAGCGAGGGCTAG (speI)
CN3120	TAGCTAAAGCTTATTTAAATCTA**CTTCTCGAACTGCGGG TGCGACCA**AGCGGCCGCCAGAATGTAGTCCAGGCGGGT CGC (hindIII)

[a]Restriction sites are underlined. The sequence encoding the Strep-Tag II is in bold. Mutations are italicized and bold. The ribosome binding site is lowercase..

Construction of Mutants of *M. tuberculosis*

Mutants were generated through a plasmid-based homologous recombination scheme as described (34) with some modifications. *M. tuberculosis* mc^{2}6230 and *M. tuberculosis* H37Rv were transformed with knockout vector pML1649 and grown at 37°C on 7H9/OADC/Hyg medium (with additional casamino acids and pantothenate for mc^{2}6230 strains). Transformants were picked, grown in liquid cultures and plated directly for double-cross-over mutants (DCOs). DCO candidates were screened for the presence of *xylE* and *gfp;* DCO candidates were GFP positive and XylE negative. DCO candidates were then grown in liquid culture for approximately 5 days to prepare chromosomal DNA, at which point correct candidates were confirmed by Southern blot analysis. Marked mutants (Δ*mmcO*::*loxP-gfp*$_m^{2+}$*-hyg-loxP*, abbreviated Δ*mmcO*::*hyg*) in *M. tuberculosis* mc^{2}6230 and *M. tuberculosis* H37Rv were designated ML1221 and ML1223 respectively. (The gfp variant used, *gfp*$_m^{2+}$, is codon adapted for expression in mycobacteria (75)). The Cre recombinase expression vector pCreSacB1 (a kind gift from Dr. Adrie Steyn) was used to excise the *loxP*-flanked *gfp*$_m^{2+}$*-hyg* cassette from the chromosomes of the *mmcO* DCOs. Unmarked deletion mutants were screened for loss of *gfp*. Strains were cured of pCreSacB1 by growth on 7H10/OADC plates containing 2% sucrose and incubated at 37°C. Single colonies were streaked in parallel on 7H10/OADC, 7H10/OADC/kan, and 7H10/OADC/hyg plates to confirm the loss of *hyg* and pCreSacB1. The unmarked *mmcO* (Δ*mmcO*::loxP, abbreviated Δ*mmcO*) deletion mutants

in *M. tuberculosis* mc^{2}6230 and *M. tuberculosis* H37Rv were named ML1222 and ML1224 (Table 1), respectively. Mutants were complemented with replicative vectors pML1641, pML1252, or pML1262, where indicated.

Southern Blot Analysis

Chromosomal DNA was extracted from wt and mutant strains according to standard protocols (35), subsequently 5 μg was digested with *XmaI*. Digested genomic DNA was separated on a 1% agarose gel; the gel was subsequently prepared and transferred according to standard protocols (36). The DNA was cross-linked to the membrane using a UV-crosslinker (240,000 μJ) and prehybridized for 3 h at 42°C in Dig-Easy hybridization solution (Roche). For analysis of the wt and mutant genomic regions, a probe was generated by PCR from wt genomic DNA using the primer pair CN1702/CN1703 and labelled using the PCR DIG labelling mix (Roche). Hybridization was carried out in the presence of 250 ng of digoxigenin-labeled PCR fragment at 50°C overnight. The membrane was washed and the hybridized digoxigenin-labeled probe was detected with an HRP-conjugated anti-digoxigenin antibody following the recommendations of the manufacturer (Roche). An imaging system and the software LabWorks (UVP) were used to visualize the luminescence of blots. The software Gimp2.0 was used to adjust the contrast of images.

Protein Overexpression, Purification, and Antibody Production

Protein for antibody production was overexpressed from pML1231 in *E. coli* strain BL21 (DE3). Plasmid pML1231 expresses $mmcO_{\Delta 2\text{-}35}$ from a T7 promoter; the

TAT secretion signal and putative lipidation site were removed to avoid complications from insufficient secretion or membrane association. Cells were grown to OD_{600} 0.6 and expression was induced by the addition of 1mM IPTG for 3 hours. After lysis, protein was present and nearly pure as inclusion bodies. Protein was purified using the Strep-Tag (II) and Strep-tactin Sepharose (IBA Lifesciences). Polyclonal antiserum was produced in rabbits using the adjuvant Titermax (Open Biosystems).

Anaylsis of Proteins by Gel Electrophoresis and Western Blot

Cell lysates of *M. tuberculosis* were analyzed by separation on 10% SDS-PAGE and subsequent staining with Coommassie Blue G-250 stain or by transfer and Western blot. Protein sample loading buffer contained 1% β-mercaptoethanol to ensure reduction of cysteines. MmcO was detected using polyclonal anti-serum (This study) and, where indicated, RNA polymerase (RNAP) was used as loading control and detected with mono-clonal antibody clone 8RB13 (Neoclone). Goat anti-rabbit and goat anti-mouse (Sigma) secondary antibodies conjugated to horseradish peroxidase were used. Blots were visualized with ECL Western blotting substrate (Pierce). An imaging system and the software LabWorks (UVP) were used to visualize and quantify the luminescence of blots. The software Gimp2.0 was used to adjust the contrast of images.

Induction of *mmcO* Expression in *M. tuberculosis* by Copper

M. tuberculosis $mc^2$6230 was grown to OD_{600} 1.0 in liquid culture. Then, cultures were split and either left un-induced or induced with 200 μM $CuSO_4$ for an additional 48 hours. Cells were collected, washed twice with PBS 0.02% Tyloxapol and resuspended in

5 μL PBS 1% SDS buffer per mg wet cell pellet. Cells were lysed by sonication and boiled with protein sample loading buffer before SDS-PAGE and Western blot analysis as above.

Whole Cell Lysate Oxidase Assays

Oxidase assays were performed as described with modifications (20, 37). *M. tuberculosis* $mc^2$6230 and derivatives were grown in media as above to approximately OD_{600} 1.0. Cells were collected and washed twice with 100mM Tris pH 7.8 100mM NaCl 0.02% Tyloxapol and resuspended in the same buffer at 5 μL buffer per mg wet cell pellet. Cells were lysed by sonication and lysate was cleared by centrifugation 5 min at 16000 g. Cleared supernatent was used in oxidase assays. The oxidase reaction mix contained 50 mM sodium acetate pH 5.5, 250 μM $CuSO_4$, 10 μL cell lysate and 20 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or 2 mM dimethoxyphenol (DMP) or 2 mM para-phenylenediamine (pPD) as substrates. Reactions (200 μL) were mixed in 96-well clear plastic, flat-bottom microplates (Costar) sealed with clear polypropylene film (USA Scientific) and monitored by microplate reader (Biotek Synergy HT). Reactions proceeded at room temperature (~23°C) with shaking and absorbance was read every 5 min for up to 8 hours. Change in absorbance of ABTS, DMP and pPD was monitored at 436 nm, 570 nm and 468 nm, respectively. Ferroxidase assays were conducted as above with 200 μM $FeSO_4$ as the substrate. Fe(II) was injected into wells and oxidation was monitored by increase of absorbance at 315 nm (38) over 15 min, before precipitates formed. Reaction absorbance in all cases was corrected by subtracting absorbance of substrate mix without lysate; samples were further normalized

by protein concentration of lysate which was determined by Bradford assay (Pierce). Oxidation rates of ABTS were determined using the extinction coefficient of oxidized ABTS at 436 nm, ε 29300 M^{-1} cm^{-1} (20). The kinetics of Fe(II) oxidation were determined using the extinction coefficient at 315 nm ε_{315} = 2200 M^{-1} cm^{-1} (38). Oxidation rates are expressed as μM min^{-1} mg^{-1} (ABTS oxidation) or mM min^{-1} mg^{-1} (Fe(II) oxidation) where mg represents the mass of total protein in the water soluble fraction used in the oxidase assays.

Copper Susceptibility of *M. tuberculosis*

Drop assays to determine copper susceptibility were performed as described (4). Briefly, *M. tuberculosis* $mc^2$6230 and derivatives or *M. tuberculosis* H37Rv and derivatives were grown in media as above to approximately OD_{600} 2.0, washed in PBS pH 7.4 0.02% Tyloxapol and serially diluted in 10-fold increments in the same buffer, 3 μL drops were spotted onto plates (media as above) containing increasing concentrations of $CuSO_4$. Plates were incubated at 37°C for 10-12 days until single colonies were visible at the lowest dilutions.

Subcellular Fractionation of *M. tuberculosis*

Protein localization was determined by subcellular fractionation as described (34). Briefly, *M. tuberculosis* $mc^2$6230 was grown in liquid culture, cells were collected and washed in PBS, and resuspended in PBS 1mM PMSF at 4 μL per mg wet cell pellet. Samples were lysed by sonication and incubated with 1 mg/mL lysozyme plus 15 U benzonase at 37°C to complete cell wall and chromosomal DNA digestion. Unbroken

cells were removed by centrifugation at 3200 g. Samples were diluted five-fold and centrifuged at 120000 g for 1 h, pellet (P100.1) and supernatant (S100.1) were separated, pellet was resuspend and centrifugation was repeated on both samples. The final pellet (P100.2, membrane fraction) and supernatant (S100.2, cytosolic fraction) along with cleared cell lysate were analysed by SDS-PAGE and Western blot as above. Antibodies against IdeR (39) and GlpX (obtained from Dr. Axel Siroy) were used as markers for cytosolic proteins, and antibody against Rv1698 (4) was used as a marker of the membrane fraction.

RESULTS

The MmcO Protein is Highly Conserved in Pathogenic Mycobacteria

MmcO (Rv0846c) was predicted as a multicopper oxidase based on conservation of active site residues and homology to other multicopper oxidases (40). MmcO shares homology with multicopper oxidases throughout the order *Actinomycetales* and is conserved among almost all pathogenic mycobacteria with the notable exception of *M. leprae,* which contains only a pseudogene (Figure S1). MmcO shares between 69 and 99% amino acid sequence identity with its homologs in other pathogenic mycobacteria such as *M. bovis* and *M. marinum*. MmcO is over 40% identical to its homologs in *Corynebacterium glutamicum* and *C. diphtheria*. Interestingly, there is no multicopper oxidase annotated in the non-pathogenic strains *M. smegmatis*, *M. chelonae,* and *M. terrae.*

Generation of an *M. tuberculosis* Δ*mmcO* Mutant

To examine the role of MmcO in copper resistance in *M. tuberculosis* we generated *mmcO* deletion mutants by homologous recombination. The gene *mmcO* does not belong to an operon. Since the surrounding genes are transcribed in the opposite direction, disruption of *mmcO* is unlikely to affect transcription of other genes. Marked double cross-over mutants (Δ*mmcO*::*loxP-gfp*$^{2+}{}_{m}$-*hyg-loxP*, abbreviated Δ*mmcO*::*hyg*) were selected by hygromycin resistance and screened for GFP fluorescence. Mutants were subsequently unmarked, indicated by the loss of these markers, using an expression vector encoding Cre-recombinase. We generated mutants in both the virulent *M. tuberculosis* strain H37Rv and the avirulent strain mc^{2}6230. The avirulent mutant

mc^{2}6230 contains deletions of the RD1 region as well as pantothenate biosynthesis genes *panCD* (32). The avirulent strain had the same copper susceptibility as its virulent parent strain H37Rv and was used primarily for biochemical assays. Marked mutants (Δ*mmcO*::*hyg*) and unmarked mutants (Δ*mmcO::loxP*, abbreviated Δ*mmcO*) in *M. tuberculosis* mc^{2}6230 and *M. tuberculosis* H37Rv (unmarked only) were confirmed by Southern blot (Figures 1A and B). The deletion strategy employed replaced the *mmcO* gene, except 13 bp at the 3' end, with a single loxP site. Loss of MmcO in *M. tuberculosis* mc^{2}6230 was confirmed by Western blot (Figure 1C).

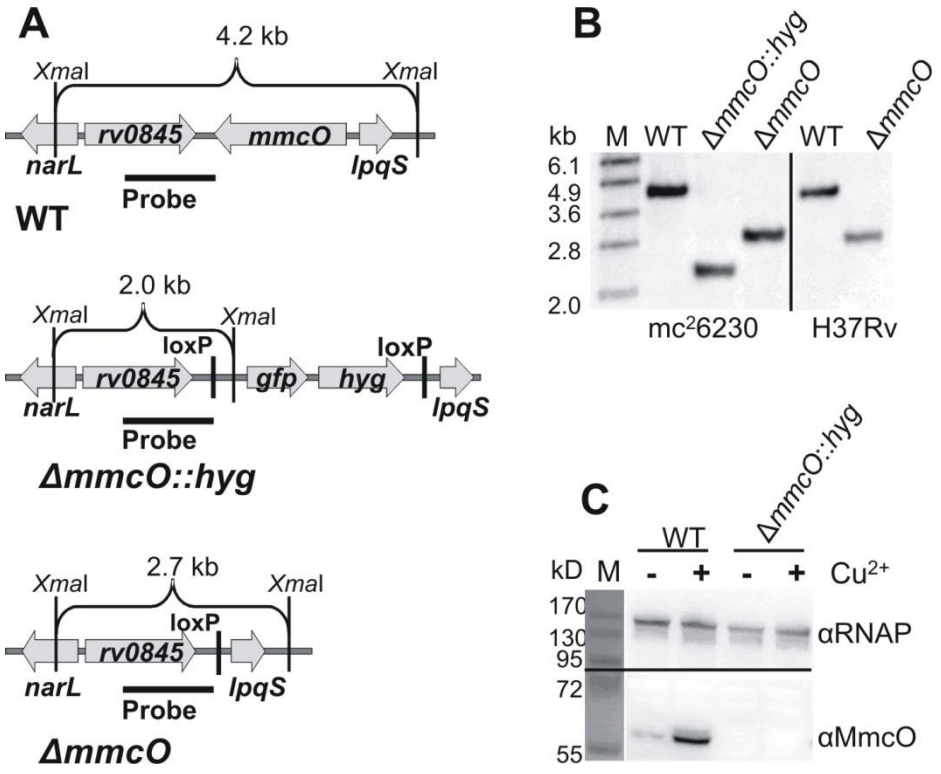

Figure 1. **Generation of an *M. tuberculosis* Δ*mmcO* mutant. A**. Depiction of genomic regions of *M. tuberculosis* wild-type (wt), Δ*mmcO*::*hyg* (marked mutant) and Δ*mmcO* (unmarked mutant). **B**. Southern blot of genomic DNA from strains depicted in A digested with XmaI. The location of probe is shown in A. Mutants were made in both the avirulent *M. tuberculosis* strain mc^{2}6230 and virulent *M. tuberculosis* H37Rv. **C**. Western blot of proteins in lysates of the mc^{2}6230 and Δ*mmcO*::*hyg* strains grown in

cultures with (+) and without (-) 200 μM $CuSO_4$. MmcO protein was detected with αMmcO antiserum. RNA polymerase (RNAP) was used as a loading control and was detected with a monoclonal antibody.

Expression of t*he mmcO* Gene is Increased by Copper

Previously, quantitative RT-PCR experiments showed that mRNA levels of *mmcO* are increased by addition of 50 μM copper in Sauton's medium (41). Expression of the *mmcO* gene is regulated by the copper responsive transcription regulator RicR (42). To examine how MmcO protein levels changed in *M. tuberculosis* under copper stress, we utilized the avirulent *M. tuberculosis* $mc^2$6230 strain and its isogenic Δ*mmcO* mutant. A single band with the apparent molecular mass of 56 kD, the predicted size of MmcO, was detected by Western blot in the wt *M. tuberculosis* untreated sample (Figure 1C). MmcO protein levels increased over basal expression levels by six-fold when 200 μM copper was added to the medium (7H9 supplemented with OADC, pantothenate and casamino acids) (Figure 1C). In addition to the dominant band with the same electrophoretic mobility as basal MmcO, a faint band with a higher apparent molecular mass was observed in the copper induced sample. MmcO contains a Twin-Arginine Translocation (TAT) signal at the N-terminus (43). Previous reports showed that the TAT secretion system is more easily saturated than the Sec secretion system leading to incomplete transport and processing of TAT substrate proteins (44, 45). Thus, the additional band might result from unprocessed MmcO in copper-stressed *M. tuberculosis* due to incomplete translocation and processing of the TAT secretion signal when the *mmcO* gene is overexpressed.

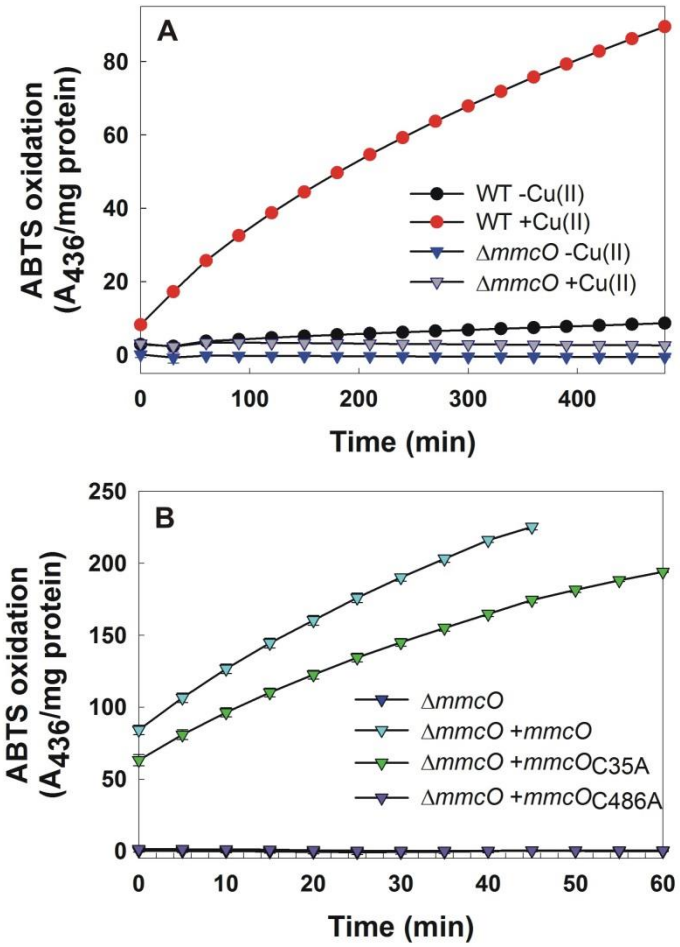

Figure 2. *M. tuberculosis* has multicopper oxidase activity. A. Oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) by wt *M. tuberculosis* $mc^2$6230 and the Δ*mmcO* mutant. Cultures were grown and treated with 200 μM copper for 48 hours prior to lysis or left untreated. **B**. ABTS oxidation by MmcO, $MmcO_{C35A}$ or $MmcO_{C486A}$ in cell lysates of the Δ*mmcO* mutant. Cultures were grown in the absence of additional copper. Assays were performed in triplicate and error bars represent standard deviations.

MmcO is a Multicopper Oxidase

We used *in vitro* oxidation assays to examine whether MmcO has the predicted multicopper oxidase activity. Several attempts to obtain soluble, functional MmcO from a variety of systems and conditions failed. Expression of *mmcO* with or without TAT signal sequence under the control of the T7 promoter in *E. coli* BL21 (DE3), regardless

of temperature during growth or induction, or addition of copper to growth medium, resulted in inclusion body formation. Expression of a fusion protein of MmcO with the maltose binding protein at the N-terminus in *E. coli* also resulted in insoluble protein, possibly due to the lack of TAT signal or missing chaperones. Fusion of the *E. coli* CueO signal peptide (amino acid residues 1 to 28) with MmcO lacking its native TAT signal peptide (MmcO amino acid residues 36 to 504), with the gene fusion expressed under the control of the *cueO* native promoter, resulted in only very low levels of protein even under copper induction. The CueO-MmcO fusion protein did not complement the loss of CueO in an *E. coli* Δ*cueO* strain. Expression of *mmcO* using inducible expression systems in *M. smegmatis* (46, 47) resulted in only very low amounts of protein, which could not be purified.

Therefore, we used cleared whole cell lysates of different strains to characterize the multicopper oxidase activity in *M. tuberculosis*. To this end, we monitored oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), a widely used substrate for multicopper oxidases because of its rapid oxidation and easy photometric detection, by whole cell lysates of wt $mc^2$6230 and the Δ*mmcO* mutant (20, 37). Wild-type lysate showed slow ABTS oxidation (2 μM min^{-1} mg^{-1} total soluble protein in assay), which was 17-fold increased when cultures were exposed to copper for 48 hours before lysis (34.5 μM min^{-1} mg^{-1}) (Figure 2A). This result is consistent with the low expression of *mmcO* under standard growth conditions and six-fold increased expression when cells were copper stressed (Figure 1C). The mutant Δ*mmcO* was unable to oxidize ABTS independent of copper stress during growth (oxidation rate 0 under either condition) demonstrating MmcO (Rv0846c) is the only multicopper oxidase present in *M.*

tuberculosis under the conditions tested. Wild-type whole cell lysates also oxidized other known multicopper oxidase substrates such as para-phenylenediamine and dimethoxyphenol (data not shown). Overexpression of *mmcO* in the Δ*mmcO* mutant restored ABTS oxidation activity, which was increased compared to copper-stressed wt *M. tuberculosis* (Figure 2B). The rate of ABTS oxidation by MmcO was 588.2 μM min^{-1} mg^{-1} and 409.1 μM min^{-1} mg^{-1}, in untreated and copper treated samples, respectively. These results demonstrate that *rv0846c* encodes a copper responsive multicopper oxidase in *M. tuberculosis*. Therefore we have renamed Rv0846c mycobacterial multicopper oxidase (MmcO).

The Putative Lipidation Site is Dispensible for MmcO Activity

We investigated the role of the putative lipidation site, cysteine 35 (predicted by the LipoP server (48)), in MmcO oxidase activity. Mutation of the putative lipidation site cysteine 35 to alanine ($MmcO_{C35A}$) leaves the TAT translocation signal intact and had only a minor effect on oxidase activity (Figure 2B). ABTS was oxidized by $MmcO_{C35A}$ at 450.9 μM min^{-1} mg^{-1} or 236.8 μM min^{-1} mg^{-1} in untreated or copper treated samples, respectively, a slight reduction compared to wt MmcO. The protein levels of mutant $MmcO_{C35A}$ and wt MmcO in the *M. tuberculosis* Δ*mmcO* mutant were similar, indicating the mutation did not lead to degradation (data not shown). This result showed that lipidation of MmcO is not essential for the multicopper oxidase activity of MmcO, but rather stimulates its activity in *M. tuberculosis*.

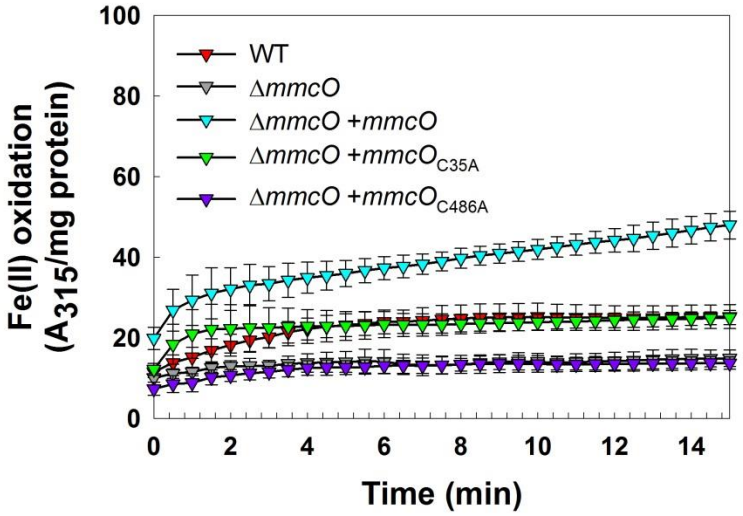

Figure 3. Fe(II) oxidation by MmcO in whole cell lysates of *M. tuberculosis*. The indicated *M. tuberculosis* strains $mc^2$6230 (wt) and derivatives were grown to OD_{600} 1.0 and treated with 200 μM copper for 48 hours prior to lysis. Oxidation of ferrous to ferric iron in cell lysates was determined by measuring the absorbance at 315 nm. The ferroxidase assay was performed in triplicate. Error bars represent standard deviations.

The Active Site Cysteine 486 is Required for MmcO Activity

Multicopper oxidases contain one cysteine residue which is critical to the coordination of the Type 1 (T1) copper, the site of substrate oxidation (27). In MmcO, the T1 copper is coordinated by Cys486, based on alignment with fully characterized multicopper oxidases (Figure S1). Mutation of the cysteine in the T1 *E. coli* CueO (C500S) resulted in protein which was expressed and fully folded but non-functional, and could be crystalized under the same conditions as wt CueO (49, 50). Similarly, the $MmcO_{C486A}$ protein failed to restore ABTS oxidation activity to the *M. tuberculosis* Δ*mmcO* mutant (Figure 2B). Like the $MmcO_{C35A}$ mutant, $MmcO_{C486A}$ expressed in Δ*mmcO* could be detected at similar levels to wt MmcO by Western blot(data not

shown). These results show that while the putative lipidation site is dispensable for activity, the active site Cys486 residue is absolutely required for substrate oxidation.

MmcO Has Ferroxidase Activity

Some multicopper oxidases can oxidize metals. Well-known multicopper oxidases Fet3P from *Saccharomyces cerevisiae* and ceruloplasmin from humans are ferrous and cuprous oxidases (51). Examples of bacterial metalloxidases include CueO, which can oxidize Fe(II) to Fe(III) and Cu(I) to Cu(II) (50) and the *Pseudomonas aeruginosa* multicopper oxidase which also oxidizes Fe(II) to Fe(III) (52). Thus, we examined whether MmcO had ferroxidase activity similar to other MCOs. Cultures of wt *M. tuberculosis*, the Δ*mmcO* mutant and the complemented strains were grown in 7H9 medium with supplements (OADC, casamino acids and pantothenate) and exposed to 200 μM copper prior to lysis. Oxidation of Fe(II) by whole cell lysates was monitored by absorbance at 315 nm, which reflects the appearance of Fe(III) in solution (38). Because Fe(III) quickly forms insoluble particles in solution, the ferroxidase assay was stopped after 15 minutes, when such particles appeared. The parental strain *M.tuberculosis* $mc^2$6230 and the Δ*mmcO* mutant expressing *mmcO* or $mmcO_{C35A}$ had ferroxidase activity (Figure 3). Ferroxidase activity was increased in cultures exposed to copper before lysis compared to untreated cells (data not shown). Wild-type *M. tuberculosis* $mc^2$6230 lysates oxidized Fe(II) at a rate of 6.6 mM min^{-1} mg^{-1}. Overexpression of *mmcO* and $mmcO_{C35A}$ in the Δ*mmcO* strain resulted in ferroxidase rates of 21.6 and 19.9 mM min^{-1} mg^{-1}, respectively. Similar to oxidation of ABTS, loss of the lipidation site cysteine 35 only slightly decreased oxidase activity compared to wt MmcO. The Δ*mmcO* mutant slowly

oxidized Fe(II) with a rate of 3.6 mM min^{-1} mg^{-1}, but oxidation activity did not reach the maximum achieved by wt lysates (Figure 3). It is possible that other oxidases or excess copper in the whole cell lysates of the Δ*mmcO* mutant resulted in slightly increased oxidation of Fe(II) over air background. Expression of $mmcO_{C486A}$ in the Δ*mmcO* mutant did not significantly alter Fe(II) oxidation compared to that obtained with the lysate of the Δ*mmcO* strain. These results demonstrate that MmcO has iron oxidation capacity in addition to ABTS and phenol oxidase activity and that the active site residue cysteine 486 is required for this catalytic activity.

MmcO is Required for Copper Resistance in *M. tuberculosis*

Multicopper oxidases are required for copper resistance in several bacterial species (20-22, 24). Thus, we hypothesized that MmcO may also be required for copper resistance in *M. tuberculosis*. We used a serial dilution assay on 7H10 with OADC (H37Rv) or 7H10 with OADC and supplemented with pantothenate and casamino acids ($mc^2$6230) containing increasing amounts of copper to determine the copper susceptibility of Δ*mmcO* mutants. Mutant strains did not show any growth defects on standard 7H10 medium (Figures 4A, B and C) in agreement with previous results that MmcO is not required for *in vitro* growth under standard conditions by Transposon Site Hybridization (TraSH) (53). However, the Δ*mmcO* mutant was susceptible to copper at 50 μM in solid medium (Figure 4A) while wt grew normally at copper concentrations up to 75 μM (Figure 4B). Overexpression of *mmcO* or $mmcO_{C35A}$ restored growth of Δ*mmcO* at 50 μM copper; however, $mmcO_{C486A}$ only slightly complemented growth (Figure 4A). Additionally we compared the Δ*mmcO* mutant to our previously

characterized copper-susceptible mutant Δ*rv1698* (4). The Δ*mmcO* mutant was highly susceptible to 75 μM copper while Δ*rv1698* grew normally (Figure 4B), consistent with previous findings (4).

To confirm that copper susceptibility was the direct result of loss of MmcO, and not from an unknown interaction between MmcO and components of RD1 or pantothenate biosynthesis in the avirulent *M. tuberculosis* strain $mc^2$6230, we repeated the experiment using H37Rv and its isogenic mutant ML1224 (H37Rv Δ*mmcO*, Table 1). Deletion of *mmcO* in H37Rv resulted in copper susceptibility similar to results obtained in *M. tuberculosis* $mc^2$6230 (Figure 4C). *M. tuberculosis* H37Rv and H37Rv Δ*mmcO* grew equally well on standard 7H10 medium with OADC, which contains 6.3 μM copper. However, Δ*mmcO* had a severe growth defect on plates containing 50 μM copper and did not grow on plates containing 75 μM copper. These results show that MmcO is required for copper resistance in *M. tuberculosis*, and that this activity is dependent on a functional active site (Cys486) and independent of the putative lipidation site Cys35.

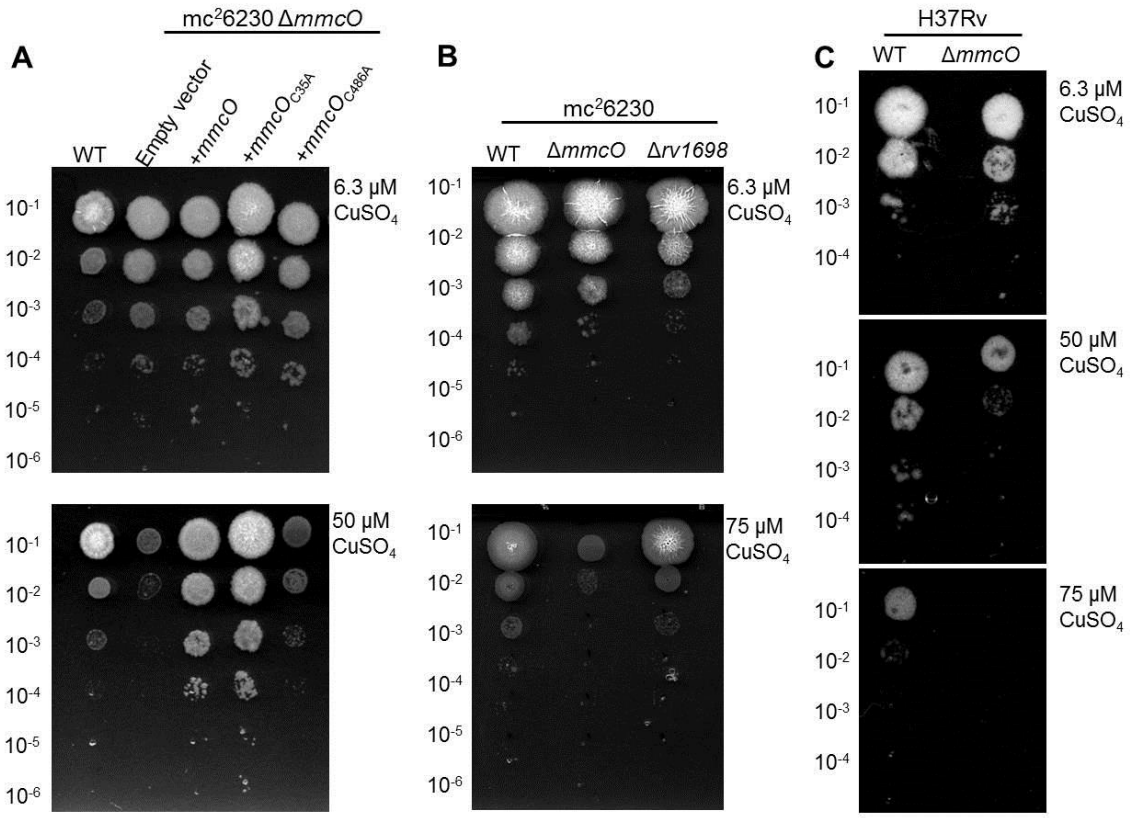

Figure 4. MmcO is required for copper resistance in *M. tuberculosis*. Liquid cultures of strains were grown to ~OD_{600} 2.0 washed, normalized and serially diluted. **A**. Drops of 3 μL of 10^{-1} to 10^{-6} dilutions of *M. tuberculosis* mc^26230 and derivatives were spotted onto Middlebrook 7H10/OADC agar plates containing casamino acids, pantothenate and 6.3 μM $CuSO_4$ or 50 μM $CuSO_4$. The experiment was performed twice and representative data are shown. **B**. Growth of the *M. tuberculosis* strains mc^26230, Δ*mmcO* and Δ*rv1698* on Middlebrook 7H10/OADC agar plates containing casamino acids, pantothenate and 6.3 or 75 μM $CuSO_4$. **C**. Serial dilutions of wt *M. tuberculosis* H37Rv and its corresponding Δ*mmcO* mutant were spotted onto Middlebrook 7H10/OADC agar plates containing 6.3, 50 or 75 μM $CuSO_4$.

MmcO is Associated with Membranes

Many multicopper oxidases are membrane associated including eukaryotic Fet3p of *S. cerevisiae* and hephaestin in humans, and CotA of *Bacillus subtillis* (54). However, periplasmic multicopper oxidases of Gram-negative bacteria (CueO of *E. coli* and the

multicopper oxidase of *Campylobacter jejuni*) are not membrane associated (24, 45). MmcO carries a potential lipidation site at Cys35 and is secreted by the TAT secretion system (43), indicating this protein may be membrane associated in the periplasm of *M. tuberculosis*. We characterized the localization of MmcO by subcellular fractionation. Separation of the membrane fraction from the cytosol and soluble periplasmic fraction was monitored with the marker proteins Rv1698 (membrane) and GlpX and IdeR (cytoplasm) (Figure 5). Seventy percent of MmcO was membrane-associated (Figure 5). The incomplete fractionation pattern may be an overexpression artifact as observed before (Figure 1C).

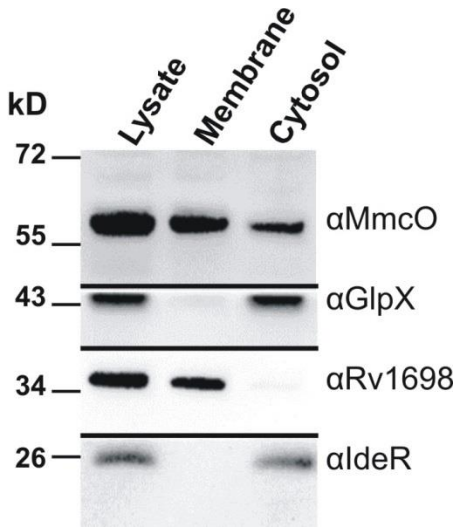

Figure 5. MmcO is membrane associated in *M. tuberculosis*. *M. tuberculosis* mc^{2}6230 cells were lysed and fractionated by ultra-centrifugation to separate water-soluble from membrane proteins. GlpX and IdeR were used as controls for water-soluble proteins and Rv1698 was used as a marker for membrane proteins.

DISCUSSION

MmcO is Required for Copper Resistance in *M. tuberculosis*

Deletion of the *mmcO* gene results in at least 10-fold increased sensitivity to 50 μM copper compared to wild-type *M. tuberculosis*. Lack of the copper metallothionein MymT or Rv1698 increases the susceptibility of *M. tuberculosis* to 150 μM copper (4, 55). By contrast, the putative copper efflux pump CtpV had only a minor effect on copper resistance in *M. tuberculosis* (56), possibly due to redundancy with another P-type ATPase, CtpG, which is induced under copper stress in the *M. tuberculosis* Δ*ctpV* mutant (56). Direct comparison revealed that the *M. tuberculosis* Δ*mmcO* mutant was more susceptible to copper than Δ*rv1698* mutant under the same conditions. These results indicate that MmcO is a major component of copper resistance of *M. tuberculosis*.

Comparison between the different mutants is difficult because the published experiments were performed under different conditions. For example, liquid culture copper exposure and subsequent colony-forming unit counts were used to determine the copper susceptibility of the *M. tuberculosis* Δ*ctpV* mutant (56). We have observed that addition of copper to liquid medium, even in the presence of copper-binding albumin causes cells to form aggregates, which makes determinations of optical density or even colony-forming unit counts difficult or error-prone (4). Further, albumin binds copper (57), and thus decreases the effective concentration of copper in the culture medium and increases the apparent minimal inhibitory concentration of copper. Finally, some experiments were performed using Middlebrook 7H11 medium which contains enzymatically digested casein, a mixture of small peptides and amino acids which are known to sequester copper, masking the true susceptibility of mutant strains to copper

(58). We found that 7H11 medium protects against copper toxicity much more than 7H10 medium supplemented with acid hydrolyzed casein, possibly due to incomplete protein digestion of enzymatically treated casein. A further complication is that the published deletion mutants were generated using different disruption methods: by transposon mutagenesis or allelic exchange with or without leaving resistance markers in place. Clearly, direct comparison of maker-free *M. tuberculosis* mutants with in-frame deletions of copper homeostasis genes, under the same conditions, preferably using medium without albumin and amino acids, is required to ultimately quantify the contributions of individual proteins to the different mechanisms of copper homeostasis in *M. tuberculosis*.

MmcO is a Multicopper Oxidase with Ferroxidase Activity

Several ferroxidases, including CueO *of E. coli* and McoC of *C. jejuni*, also have cuprous oxidase activity (59), suggesting that the physiological role of MmcO might be to oxidize toxic Cu(I). Indeed, elimination of the oxidase activity of MmcO impairs its ability to protect *M. tuberculosis* against copper (Figure 4). However, siderophore oxidation has been proposed as an indirect mechanism by which bacterial MCOs could protect against copper toxicity. According to this model, a side-reaction of Fe(II)-siderophore complexes is to reduce Cu(II) to the more toxic Cu(I) and thus, CueO oxidation of Fe(II) loaded siderophores results in lower levels of Cu(I) (60). It has not been definitively determined whether CueO protects *E. coli* from Cu(I) toxicity by direct oxidation of Fe(II)-siderophores or by a combination of both mechanisms (60).

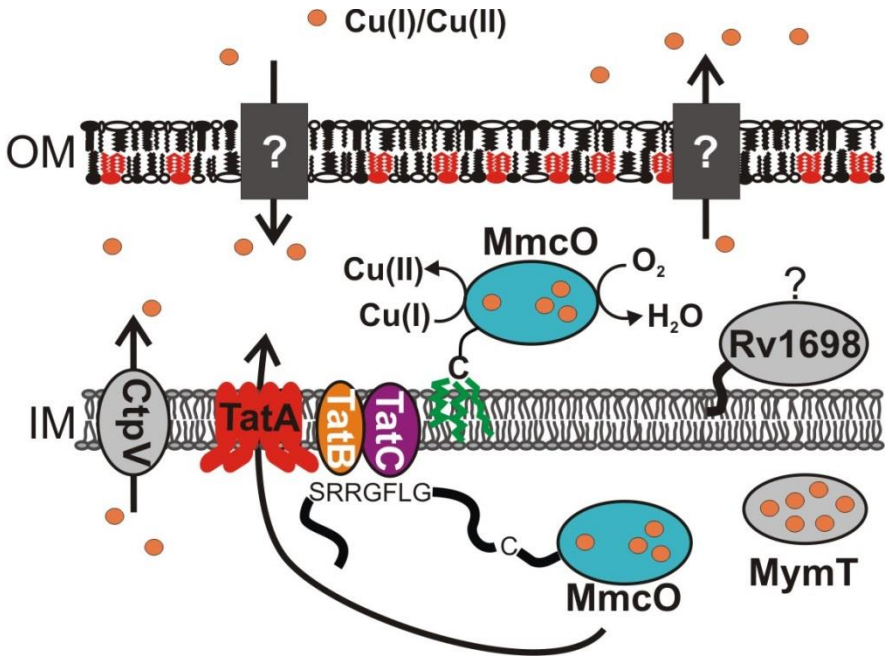

Figure 6. Model of copper homeostasis in *M. tuberculosis*. The outer membrane (OM) is an asymmetrical bilayer containing extractable lipids in both leaflets. The inner leaflet contains long chain mycolic acids (red) which are covalently linked to the arabinogalactan-peptidoglycan polymer (61). The inner membrane (IM) is a typical plasma membrane composed of phospholipids (61). Channel proteins required for copper influx and efflux across the OM are unknown. The P-type ATPase, CtpV, pumps copper out of the cytoplasm and is localized in the IM (56). MymT protects the cytoplasm against copper stress by sequestering four to six copper ions (55). TatB recognizes and binds the twin-arginine (RR) motif on the N-terminus of MmcO prior to translocation (62). MmcO is translocated to the periplasm by the Twin-Arginine Translocation system (TatABC) probably as folded protein (43). After translocation, MmcO remains membrane associated, likely through acylation (green) at cysteine 35. MmcO protects *M. tuberculosis* against copper stress, probably by oxidizing toxic Cu(I) to less toxic Cu(II) in the periplasm. Rv1698 is also membrane associated and protects *M. tuberculosis* from copper toxicity by an unknown mechanism (4).

Some multicopper oxidases, such as *E. coli* CueO and *Pseudomonas syringae* CopA, bind additional copper ions outside of the active site. The extra copper binding sites of CueO are part of a methionine-rich helix which may serve to feed copper into the substrate oxidation site (49). *M. tuberculosis* MmcO does not contain such methionine-

rich helix (Figure S1). *P. syringae* CopA, on the other hand, acts by sequestering excess copper, either by binding it in the periplasm or by binding copper in the cytoplasm and translocating it to the periplasm (21). However, the active site mutant $MmcO_{C486A}$ did not restore full copper resistance to *M. tuberculosis* demonstrating that MmcO protects *M. tuberculosis* against copper stress mainly by oxidase activity, with possible contribution from copper sequestration. It is unclear whether MmcO oxidizes Cu(I) directly or if it acts on another substrate, which then reduces or sequesters Cu(I).

Role of Lipidation of MmcO

MmcO contains a cysteine residue downstream of the Twin-Arginine Translocation signal sequence in a predicted lipidation motif. Acyl-transferases link fatty acids to a cysteine immediately after the cleavage site of the signal peptidase to convert the exported protein into a lipoprotein (63). Lipoproteins can be substrates of the Sec or TAT translocation systems (64). While periplasmic proteins in Gram-positive bacteria are lipoproteins (65), their counterparts in Gram-negative bacteria are often not acylated (66), probably because the outer membrane retains these proteins within the cell boundaries. Interestingly, many periplasmic proteins in mycobacteria contain lipoboxes, although mycobacteria have an outer membrane (67). For example the periplasmic binding proteins associated with ABC transporters are not acylated in Gram-negative bacteria, but are lipoproteins in mycobacteria (68, 69). In this study, we show that cysteine acylation of the predicted lipoprotein MmcO of *M. tuberculosis* is dispensable for its function. Subcellular fractionation of $MmcO_{C35A}$ showed that a portion of the protein is still membrane-associated (data not shown). It is unclear whether this membrane association

is transient and due to the hydrophobic helix in the uncleaved TAT signal peptide. Since the TAT signal peptide as the translocation signal is not altered, it is likely that the $MmcO_{C35A}$ mutant is localized in the periplasm as is wt MmcO. However, we cannot exclude the possibility that some $MmcO_{C35A}$ protein might be in the cytosol. Even if this were the case, it is unknown whether the usually periplasmic multicopper oxidases would be active in the cytosol. It is possible that lipidation orients the MmcO protein in the membrane or alters protein conformation such that the active site is more accessible, maybe in association with other proteins. Such a scenario would provide an explanation for the reduced oxidation activity of $MmcO_{C35A}$ compared to wt MmcO.

Role of Protein Localization in Copper Resistance of *M. tuberculosis*

MmcO and the putative MCOs of other mycobacterial species contain a Twin-Arginine Translocation signal sequence, which is utilized by bacteria to translocate folded proteins across the inner membrane (70). It is possible that MmcO binds copper in the cytoplasm and is then exported; however, there is evidence that MCOs can be folded and exported without the full complement of cofactors (49, 71-73). Fusions of the MmcO signal peptide with β-lactamase indicate that MmcO is a periplasmic protein (43), similar to several other bacterial multicopper oxidases including CueO (20), CopA of *Pseudomonas syringae* (21), and *C. jejuni* McoC (59). The putative localization of MmcO in the periplasm and its important role in copper resistance indicate that detoxification of periplasmic Cu(I) is the physiological role of MmcO in *M. tuberculosis* (Fig. 6). CtpV and MymT both protect the cytoplasm from copper stress and hence the absence of either one of those proteins may, at least partially, be compensated for by the

other protein (Fig. 6). Locational redundancy of CtpV and MymT may explain why the *M. tuberculosis* Δ*ctpV* mutant is not much more susceptible to copper than the wild-type (56). The absence of Rv1698 causes copper to accumulate in *M. tuberculosis* (4); however, its mechanism of action and its precise localization are unclear (13). Taken together, this study showed that MmcO is a principal component of copper resistance of *M. tuberculosis*. It is important to identify the missing proteins of copper homeostasis in *M. tuberculosis* (Fig. 6) and to elucidate the interplay of these mechanisms to understand how *M. tuberculosis* withstands the copper overload of the phagosome in macrophages (15). A better understanding of copper homeostasis in *M. tuberculosis* may also help to utilize copper in the development of novel anti-TB drugs, as shown recently (74).

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

REFERENCES

1. **WorldHealthOrganization.** 2013. Global tuberculosis control: WHO report 2012.

2. **Failla ML.** 2003. Trace elements and host defense: recent advances and continuing challenges. J Nutr **133:**1443S-1447S.

3. **Percival SS.** 1998. Copper and immunity. Am J Clin Nutr **67:**1064S-1068S.

4. **Wolschendorf F, Ackart D, Shrestha TB, Hascall-Dove L, Nolan S, Lamichhane G, Wang Y, Bossmann SH, Basaraba RJ, Niederweis M.** 2011. Copper resistance is essential for virulence of *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 108:1621-1626.

5. **Achard ME, Stafford SL, Bokil NJ, Chartres J, Bernhardt PV, Schembri MA, Sweet MJ, McEwan AG.** 2012. Copper redistribution in murine macrophages in response to *Salmonella* infection. Biochem J **444:**51-57.

6. **Pena MM, Lee J, Thiele DJ.** 1999. A delicate balance: homeostatic control of copper uptake and distribution. J Nutr **129:**1251-1260.

7. **Hong R, Kang TY, Michels CA, Gadura N.** 2012. Membrane lipid peroxidation in copper alloy-mediated contact killing of *Escherichia coli*. Appl Environ Microbiol **78:**1776-1784.

8. **Sellin S, Eriksson LE, Mannervik B.** 1987. Electron paramagnetic resonance study of the active site of copper-substituted human glyoxalase I. Biochemistry **26:**6779-6784.

9. **Hiniker A, Collet JF, Bardwell JC.** 2005. Copper stress causes an *in vivo* requirement for the *Escherichia coli* disulfide isomerase DsbC. J Biol Chem **280:**33785-33791.

10. **Macomber L, Imlay JA.** 2009. The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. Proc Natl Acad Sci U S A **106:**8344-8349.

11. **Dameron CT, Harrison MD.** 1998. Mechanisms for protection against copper toxicity. Am J Clin Nutr **67:**1091S-1097S.

12. **Rosen BP.** 2002. Transport and detoxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. Comp Biochem Physiol A Mol Integr Physiol **133:**689-693.

13. **Rowland JL, Niederweis M.** 2012. Resistance mechanisms of *Mycobacterium tuberculosis* against phagosomal copper overload. Tuberculosis (Edinb) **92:**202-210.

14. **Failla ML, Hopkins RG.** 1998. Is low copper status immunosuppressive? Nutr Rev **56:**S59-64.

15. **White C, Lee J, Kambe T, Fritsche K, Petris MJ.** 2009. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem **284:**33949-33956.

16. **Wagner D, Maser J, Lai B, Cai Z, Barry CE, 3rd, Honer Zu Bentrup K, Russell DG, Bermudez LE.** 2005. Elemental analysis of *Mycobacterium avium*-, *Mycobacterium tuberculosis*-, and *Mycobacterium smegmatis*-containing phagosomes indicates pathogen-induced microenvironments within the host cell's endosomal system. J Immunol **174:**1491-1500.

17. **Botella H, Stadthagen G, Lugo-Villarino G, de Chastellier C, Neyrolles O.** 2012. Metallobiology of host-pathogen interactions: an intoxicating new insight. Trends Microbiol **20:**106-112.

18. **Hodgkinson V, Petris MJ.** 2012. Copper homeostasis at the host-pathogen interface. J Biol Chem **287:**13549-13555.

19. **Samanovic MI, Ding C, Thiele DJ, Darwin KH.** 2012. Copper in microbial pathogenesis: meddling with the metal. Cell Host Microbe **11:**106-115.

20. **Grass G, Rensing C.** 2001. CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. Biochem Biophys Res Commun **286:**902-908.

21. **Cha JS, Cooksey DA.** 1991. Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. Proc Natl Acad Sci USA **88:**8915-8919.

22. **Achard ME, Tree JJ, Holden JA, Simpfendorfer KR, Wijburg OL, Strugnell RA, Schembri MA, Sweet MJ, Jennings MP, McEwan AG.** 2010. The multi-copper-ion oxidase CueO of *Salmonella enterica* serovar *Typhimurium* is required for systemic virulence. Infect Immun **78:**2312-2319.

23. **Lee YA, Hendson M, Panopoulos NJ, Schroth MN.** 1994. Molecular cloning, chromosomal mapping, and sequence analysis of copper resistance genes from *Xanthomonas campestris pv. juglandis*: homology with small blue copper proteins and multicopper oxidase. J Bacteriol **176:**173-188.

24. **Hall SJ, Hitchcock A, Butler CS, Kelly DJ.** 2008. A Multicopper oxidase (Cj1516) and a CopA homologue (Cj1161) are major components of the copper homeostasis system of *Campylobacter jejuni*. J Bacteriol **190:**8075-8085.

25. **Hsiao YM, Liu YF, Lee PY, Hsu PC, Tseng SY, Pan YC.** 2011. Functional characterization of *copA* gene encoding multicopper oxidase in *Xanthomonas campestris pv. campestris*. J Agric Food Chem **59:**9290-9302.

26. **Solomon EI, Sundaram UM, Machonkin TE.** 1996. Multicopper oxidases and oxygenases. Chem Rev **96:**2563-2606.

27. **Quintanar L, Stoj C, Taylor AB, Hart PJ, Kosman DJ, Solomon EI.** 2007. Shall we dance? How a multicopper oxidase chooses its electron transfer partner. Acc Chem Res **40:**445-452.

28. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

29. **Stephan J, Bender J, Wolschendorf F, Hoffmann C, Roth E, Mailänder C, Engelhardt H, Niederweis M.** 2005. The growth rate of *Mycobacterium smegmatis* depends on sufficient porin-mediated influx of nutrients. Mol. Microbiol. **58:**714-730.

30. **Jones CM, Niederweis M.** 2011. *Mycobacterium tuberculosis* can utilize heme as an iron source. J Bacteriol **193:**1767-1770.

31. **Bi W, Stambrook PJ.** 1998. Site-directed mutagenesis by combined chain reaction. Anal Biochem **256:**137-140.

32. **Sambandamurthy VK, Derrick SC, Hsu T, Chen B, Larsen MH, Jalapathy KV, Chen M, Kim J, Porcelli SA, Chan J, Morris SL, Jacobs WR, Jr.** 2006. *Mycobacterium tuberculosis* D*RD1* D*panCD*: a safe and limited replicating mutant strain that protects immunocompetent and immunocompromised mice against experimental tuberculosis. Vaccine 24:6309-6320.

33. **Kaps I, Ehrt S, Seeber S, Schnappinger D, Martin C, Riley LW, Niederweis M.** 2001. Energy transfer between fluorescent proteins using a co-expression system in *Mycobacterium smegmatis*. Gene **278:**115-124.

34. **Wells RM, Jones CM, Xi Z, Speer A, Danilchanka O, Doornbos KS, Sun P, Wu F, Tian C, Niederweis M.** 2013. Discovery of a siderophore export system essential for virulence of *Mycobacterium tuberculosis*. PLoS Pathog **9:**e1003120.

35. **Larsen MH, Biermann K, Tandberg S, Hsu T, Jacobs WR, Jr.** 2007. Genetic Manipulation of *Mycobacterium tuberculosis*. Curr Protoc Microbiol **Chapter 10:**Unit 10A 12.

36. **Ausubel FA, Brent R, Kingston RE, Moore DD, Seidmann JG, Smith JA, Struhl K.** 1990. Current protocols in molecular biology. Greene Publishing and Wiley-Interscience, New York.

37. **Solano F, Lucas-Elio P, Lopez-Serrano D, Fernandez E, Sanchez-Amat A.** 2001. Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins. FEMS Microbiol Lett **204:**175-181.

38. **Kim C, Lorenz WW, Hoopes JT, Dean JF.** 2001. Oxidation of phenolate siderophores by the multicopper oxidase encoded by the *Escherichia coli yacK* gene. J Bacteriol **183:**4866-4875.

39. **Dussurget O, Rodriguez M, Smith I.** 1996. An ideR mutant of *Mycobacterium smegmatis* has derepressed siderophore production and an altered oxidative-stress response. Mol Microbiol **22:**535-544.

40. **Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE, 3rd, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Barrell BG.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393:537-544.

41. **Ward SK, Hoye EA, Talaat AM.** 2008. The global responses of *Mycobacterium tuberculosis* to physiological levels of copper. J Bacteriol **190:**2939-2946.

42. **Festa RA, Jones MB, Butler-Wu S, Sinsimer D, Gerads R, Bishai WR, Peterson SN, Darwin KH.** 2011. A novel copper-responsive regulon in *Mycobacterium tuberculosis*. Mol Microbiol **79:**133-148.

43. **McDonough JA, McCann JR, Tekippe EM, Silverman JS, Rigel NW, Braunstein M.** 2008. Identification of functional Tat signal sequences in *Mycobacterium tuberculosis* proteins. J Bacteriol **190:**6428-6438.

44. **Barrett CM, Ray N, Thomas JD, Robinson C, Bolhuis A.** 2003. Quantitative export of a reporter protein, GFP, by the twin-arginine translocation pathway in *Escherichia coli*. Biochem Biophys Res Commun **304:**279-284.

45. **DeLisa MP, Lee P, Palmer T, Georgiou G.** 2004. Phage shock protein PspA of *Escherichia coli* relieves saturation of protein export via the Tat pathway. J Bacteriol **186:**366-373.

46. **Posey JE, Shinnick TM, Quinn FD.** 2006. Characterization of the twin-arginine translocase secretion system of *Mycobacterium smegmatis*. J Bacteriol **188:**1332-1340.

47. **Pandey AK, Raman S, Proff R, Joshi S, Kang CM, Rubin EJ, Husson RN, Sassetti CM.** 2009. Nitrile-inducible gene expression in mycobacteria. Tuberculosis (Edinb) **89:**12-16.

48. **Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A.** 2003. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci **12:**1652-1662.

49. **Singh SK, Roberts SA, McDevitt SF, Weichsel A, Wildner GF, Grass GB, Rensing C, Montfort WR.** 2011. Crystal structures of multicopper oxidase CueO bound to copper(I) and silver(I): functional role of a methionine-rich sequence. J Biol Chem **286:**37849-37857.

50. **Singh SK, Grass G, Rensing C, Montfort WR.** 2004. Cuprous oxidase activity of CueO from *Escherichia coli*. J Bacteriol **186:**7815-7817.

51. **Stoj C, Kosman DJ.** 2003. Cuprous oxidase activity of yeast Fet3p and human ceruloplasmin: implication for function. FEBS Lett **554:**422-426.

52. **Huston WM, Jennings MP, McEwan AG.** 2002. The multicopper oxidase of *Pseudomonas aeruginosa* is a ferroxidase with a central role in iron acquisition. Mol Microbiol **45:**1741-1750.

53. **Griffin JE, Gawronski JD, Dejesus MA, Ioerger TR, Akerley BJ, Sassetti CM.** 2011. High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. PLoS Pathog **7:**e1002251.

54. **Enguita FJ, Martins LO, Henriques AO, Carrondo MA.** 2003. Crystal structure of a bacterial endospore coat component. A laccase with enhanced thermostability properties. J Biol Chem **278:**19416-19425.

55. **Gold B, Deng H, Bryk R, Vargas D, Eliezer D, Roberts J, Jiang X, Nathan C.** 2008. Identification of a copper-binding metallothionein in pathogenic mycobacteria. Nat Chem Biol **4:**609-616.

56. **Ward SK, Abomoelak B, Hoye EA, Steinberg H, Talaat AM.** 2010. CtpV: a putative copper exporter required for full virulence of *Mycobacterium tuberculosis*. Mol Microbiol.

57. **de Wolf FA, Brett GM.** 2000. Ligand-binding proteins: their potential for application in systems for controlled delivery and uptake of ligands. Pharmacol Rev **52:**207-236.

58. **Lodyga-Chruscinska E, Micera G, Szajdzinska-Pietek E, Sanna D.** 1998. Copper(II) Complexes of Opiate-like Food Peptides. J Agric Food Chem **46:**115-118.

59. **Silva CS, Durao P, Fillat A, Lindley PF, Martins LO, Bento I.** 2012. Crystal structure of the multicopper oxidase from the pathogenic bacterium *Campylobacter jejuni CGUG11284*: characterization of a metallo-oxidase. Metallomics **4:**37-47.

60. **Grass G, Thakali K, Klebba PE, Thieme D, Muller A, Wildner GF, Rensing C.** 2004. Linkage between catecholate siderophores and the multicopper oxidase CueO in *Escherichia coli*. J Bacteriol **186:**5826-5833.

61. **Kaur D, Guerin ME, Skovierova H, Brennan PJ, Jackson M.** 2009. Biogenesis of the cell wall and other glycoconjugates of *Mycobacterium tuberculosis*. Adv Appl Microbiol **69:**23-78.

62. **Ligon LS, Hayden JD, Braunstein M.** 2012. The ins and outs of *Mycobacterium tuberculosis* protein export. Tuberculosis (Edinb) **92:**121-132.

63. **Tokuda H, Matsuyama S.** 2004. Sorting of lipoproteins to the outer membrane in *E. coli*. Biochim Biophys Acta **1693:**5-13.

64. **Hutchings MI, Palmer T, Harrington DJ, Sutcliffe IC.** 2009. Lipoprotein biogenesis in Gram-positive bacteria: knowing when to hold 'em, knowing when to fold 'em. Trends Microbiol **17:**13-21.

65. **Sutcliffe IC, Harrington DJ, Hutchings MI.** 2012. A phylum level analysis reveals lipoprotein biosynthesis to be a fundamental property of bacteria. Protein Cell **3:**163-170.

66. **Navarre WW, Schneewind O.** 1999. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev **63:**174-229.

67. **Hoffmann C, Leis A, Niederweis M, Plitzko JM, Engelhardt H.** 2008. Disclosure of the mycobacterial outer membrane: Cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. Proc. Natl. Acad. Sci. U S A **105:**3963-3967.

68. **Titgemeyer F, Amon J, Parche S, Mahfoud M, Bail J, Schlicht M, Rehm N, Hillmann D, Stephan J, Walter B, Burkovski A, Niederweis M.** 2007. A genomic view of sugar transport in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. J Bacteriol 189:5903-5915.

69. **Niederweis M.** 2008. Nutrient acquisition by mycobacteria. Microbiology **154:**679-692.

70. **Palmer T, Berks BC.** 2012. The twin-arginine translocation (Tat) protein export pathway. Nat Rev Microbiol **10:**483-496.

71. **Ducros V, Brzozowski AM, Wilson KS, Brown SH, Ostergaard P, Schneider P, Yaver DS, Pedersen AH, Davies GJ.** 1998. Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 A resolution. Nat Struct Biol **5:**310-316.

72. **Galli I, Musci G, Bonaccorsi di Patti MC.** 2004. Sequential reconstitution of copper sites in the multicopper oxidase CueO. J Biol Inorg Chem **9:**90-95.

73. **Musci G, Di Marco S, Bellenchi GC, Calabrese L.** 1996. Reconstitution of ceruloplasmin by the Cu(I)-glutathione complex. Evidence for a role of Mg^{2+} and ATP. J Biol Chem **271:**1972-1978.

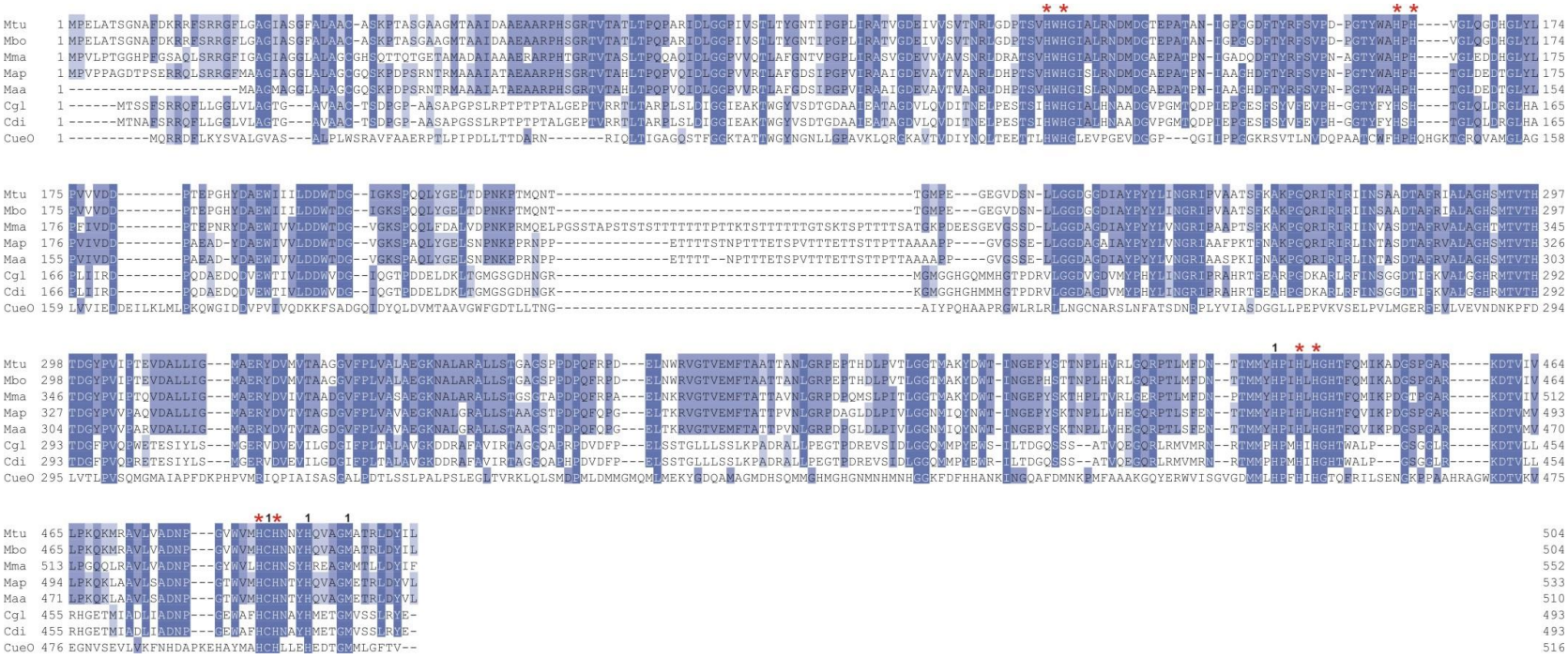
74. **Speer A, Shrestha TB, Bossmann SH, Basaraba RJ, Harber GJ, Michalek SM, Niederweis M, Kutsch O, Wolschendorf F.** 2013. Copper-boosting compounds: a novel concept for antimycobacterial drug discovery. Antimicrob Agents Chemother **57:**1089-1091.

75. **Steinhauer K, Eschenbacher I, Radischat N, Detsch C, Niederweis M, Goroncy-Bermes P.** 2010. Rapid evaluation of the mycobactericidal efficacy of disinfectants in the quantitative carrier test EN 14563 by using fluorescent Mycobacterium terrae. Appl. Environ. Microbiol. **76**:546-554.

SUPPLEMENT

Multiple sequence alignment of multicopper oxidases

Sequences of mycobacterial multicopper oxidases and others were obtained by Blast search of sequenced genomes in the NCBI database. Alignment was performed using ClustalW, and the alignment image was generated with JalView.


Mtu
Mbo
Mma
Map
Maa
Cgl
Cdi
CueO

Supplemental Figure 1. Alignment of putative MCOs in mycobacteria and other bacteria. Mtu: *M. tuberculosis*, MmcO; Mbo: *M. bovis*, Mb0869c; Mma: *M. marinum*, MMAR_4770 ; Map: *M. avium paratuberculosis* K10, MAP0701c ; Maa: *M. avium avium*, MaviaA2_3747 ; Cgl: *Corynebacterium glutamicum*, CopO (cg3287 or NCgl2865); Cdi: *C. diphtheriae*, CDHC02_0060; CueO: *E. coli* CueO. Red stars indicate conserved active site residues (Type II or Type III copper coordingating residues); bold 1 indicates conserved Type I copper coordinating residue.

PORINS INCREASE COPPER SUSCEPTIBILITY OF *MYCOBACTERIUM TUBERCULOSIS*

by

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ABSTRACT

Copper resistance mechanisms are crucial for many pathogenic bacteria, including *Mycobacterium tuberculosis*, during infection because the innate immune system utilizes copper ions to kill bacterial intruders. Despite several studies detailing responses of mycobacteria to copper, the pathways by which copper ions cross the mycobacterial cell envelope are unknown. Deletion of porin genes in *Mycobacterium smegmatis* leads to a severe growth defect on trace copper medium but simultaneously increases tolerance for copper at elevated concentrations, indicating that porins mediate copper uptake across the outer membrane. Heterologous expression of the mycobacterial porin gene *mspA* reduced growth of *M. tuberculosis* in the presence of 2.5 μM copper by 40% and completely suppressed growth at 15 μM copper while, wild-type *M. tuberculosis* reached its normal cell density at that copper concentration. Moreover, the polyamine spermine, a known inhibitor of porin activity in Gram-negative bacteria, enhanced tolerance of *M. tuberculosis* for copper suggesting that copper ions utilize endogenous outer membrane channel proteins of *M. tuberculosis* to gain access to interior cellular compartments. In summary, these findings highlight the outer membrane as the first barrier against copper ions and the role of porins in mediating copper uptake in *M. smegmatis* and *M. tuberculosis*.

INTRODUCTION

Copper is an important micronutrient and participates in essential metabolic functions in most cells. It is a crucial co-factor of heme-copper oxidases, which are found in the respiratory pathway of oxygen consuming bacteria and in eukaryotes (1). In *Mycobacterium tuberculosis*, the activity of the heme-copper oxidase is essential for *in vitro* growth (2). Furthermore, copper ions are co-factors of periplasmic or surface anchored superoxide dismutases (3) and multicopper oxidases (4). Pathogenic bacteria, including *M. tuberculosis*, *Staphylococcus aureus*, and *Burkholderia pseudomallei*, likely utilize Cu,Zn superoxide dismutases to combat the oxidative burst generated by the host's innate immune system (5-7). Multicopper oxidases have been associated with virulence of *Salmonella enterica* (8) but also contribute to copper tolerance in *Escherichia coli* (9) and *M. tuberculosis* (10), and iron acquisition by *Pseudomonas aeruginosa* (11). While copper in small amounts is beneficial, higher concentrations are toxic. Hence, microbes have evolved resistance mechanisms to maintain copper homeostasis over a broad concentration range (reviewed in reference (12)). More recently it has become clear that copper resistance is not only important for environmental bacteria, but also for pathogenic microbes. For example, *Streptococcus pneumoniae* (13), *P. aeruginosa* (14) and *M. tuberculosis* (15, 16) require copper resistance mechanisms for full virulence. The link between copper resistance and virulence is very plausible, as copper poisoning has emerged as a strategy by which macrophages kill phagocytosed bacteria (17, 18).

M. tuberculosis is equipped with at least two copper-responsive repressors, CsoR (19) and RicR (20). CsoR regulates its own expression and is encoded in an operon with the putative copper efflux pump CtpV (19). The RicR regulon includes the genes

encoding a mycobacterium-specific cytoplasmic copper metallothioneine (MymT) and a periplasmic multicopper oxidase (MmcO) (10, 20, 21). Although copper resistance pathways are of great interest (reviewed in (22-24)), little has been done to investigate how copper enters bacterial cells to be utilized metabolically or to exert its bactericidal properties.

Porins are believed to be the most likely pathway for copper uptake in Gram-negative bacteria (12). This hypothesis is largely based on a study from the year 1977 by Lutkenhaus, who described the isolation of porin-deficient copper-resistant *E. coli* mutants on copper-rich minimal medium (25). In contrast, isogenic porin mutants of *E. coli* lacking the general porins OmpF and/or OmpC, showed no difference in copper resistance (26) or were even more susceptible to copper (27) contradicting the previous interpretation of Lutkenhaus' study.

Nevertheless, copper ions, which are small and hydrophilic, could utilize the porin pathway to enter bacterial cells (28). In mycobacteria, the only known and characterized porins are MspA, MspB, MspC and MspD from *M. smegmatis* (29, 30). These porins are very similar to each other; MspB, MspC and MspD diverge only in 2, 4 and 18 amino acids, respectively, from MspA (31). However, the homo-octameric structure of Msp porins, forming one central channel, differs considerably from the homotrimeric structure of porins from Gram-negative bacteria, where each subunit forms one channel (29).

The existence of porins in *M. tuberculosis* has been demonstrated previously (32, 33), but specific porin genes are still unidentified. We hypothesized that copper uptake in mycobacteria is a porin-mediated process. We found that the porin MspA and its paralogues are essential for the acquisition of copper, especially at low copper

concentrations. Further, we establish the mycobacterial outer membrane as an efficient diffusion barrier for toxic copper ions and demonstrate that copper susceptibility of *M. smegmatis* and *M. tuberculosis* is a function of the porin expression level and the porin type. These results may have important implications for the role of putative porins in the pathogenicity of *M. tuberculosis*.

MATERIALS AND METHODS

Chemicals, Strains and Growth Conditions

All *M. smegmatis* strains and *M. tuberculosis* mc^{2}6230 (ΔRD1, Δ*panCD*) (34) were routinely grown on Middlebrook 7H10 medium (BD) or in Middlebrook 7H9 broth supplemented with 0.02% tyloxapol. Additionally, 10% oleic acid-albumin-dextrose-catalase (OADC) and 24 μg/mL pantothenate were added to support the growth of *M. tuberculosis* mc^{2}6230 (34). Trace copper versions of Middlebrook and Hartmans-de Bont (HdB) medium were prepared as previously described (15). Copper was supplied in the form of copper sulfate. The construction of the *M. smegmatis* SMR5 porin mutants MN01 (Δ*mspA*), ML10 (Δ*mspA,* Δ*mspC*) and ML16 (Δ*mspA,* Δ*mspC,* Δ*mspD*) has been published previously (30, 31, 35). Control strains carried the empty mycobacterial expression vector pMS2 (36). The pMS2 derived vectors pMN013 (p_{imyc}*mspA*) and pMN016 (p_{smyc}*mspA*) were used for the expression of *mspA* in *M. tuberculosis* and *M. smegmatis*, respectively (30, 37). The isogenic plasmids pMN041, pMN042, pMN043 (30) were used to express *mspA*, *mspB* or *mspC* in ML10. Hygromycin B (50 μg/ml) was added to all media as required. Spermine, ampicillin, copper sulfate and all media constituents were purchased from Sigma. Noble agar (BD) was used for self-made Middlebrook 7H10 plates. Hygromycin was purchased from Calbiochem and alamarBlue reagent from AbD Serotec.

Bacterial Drop Assay

The drop assay was performed as previously described (15). Briefly, *M. smegmatis* strains were grown over night in self-made trace copper 7H9 medium.

Cultures were filtered through a 5-µm filter disk to remove clumps and then adjusted to an optical density at 600 nm (OD_{600}) of 0.1. The OD-adjusted cell suspensions were then further diluted in 10-fold increments to an OD_{600} of $1x\ 10^{-6}$, and 5 µL of each of these serial cell dilutions was then spotted on self-made 7H10 plates containing trace, 25 or 100 µM copper. Plates were incubated for 3 to 5 days at 37°C and scanned using an Epson V700 scanner for documentation. To obtain individual colonies, 10 µL of the lowest dilution was plated on self-made 7H10 plates containing trace amounts or 6, 12.5, 25, 50 or 100 µM copper. The plates were incubated at 37 °C for 4 days. Pictures of individual colonies were taken using a Zeiss microscope (Stemi 2000-C) equipped with a Zeiss camera (Axiocam MRc) at 8- or 25-fold magnification.

Growth Curves of *M. tuberculosis*

Precultures of *M. tuberculosis* were grown in vented tissue culture flasks at 37°C using HdB medium without agitation. The flasks were manually shaken once a day. If necessary, clumps were broken by mild sonication in a sonication bath (FS60H; Fisher). At mid-exponential phase (OD_{600}, ~1.0), the cells were centrifuged (Eppendorf centrifuge 5810R; Rotor A-4-81; 3,000 × g; room temperature for 10 min), and each cell pellet was resuspended in 300 ml fresh HdB medium to give an OD_{600} of 0.05. Then the cells were split in 2 150-ml cultures. Copper sulfate was added to one of the duplicate cultures to a final concentration of 15 µM. Then, the 150 ml cultures were subdivided once more into 3 50-ml cultures to obtain triplicates and cultured without agitation in T75 tissue culture flasks. All cultures were treated equally during the entire duration of the experiment.

MmcO Induction Assay

Strains were grown in 10 ml HdB medium containing only trace amounts of copper ions (<1µM) (15). At an OD_{600} of 1.0, copper sulfate was added to the individual cultures to a final concentration of 2.5, 7.5, 10, 15, 20 or 30 µM. After 24 h, cells were harvested by centrifugation for 10 min at 3,000 x g and 4°C (Eppendorf centrifuge 5810R; Rotor A-4-81), resuspended in 600 µl phosphate-buffered saline (PBS) plus 2% SDS and lysed via bead beating using 0.1 mm glass beads (rpi Corp.), and the proteins were solubilized by heating at 95°C for 10 min. The insoluble debris and glass beads were removed by centrifugation at 16,000 x g. The protein content of each sample was determined spectroscopically by its A_{260}/A_{280} ratio using a UV-visible spectrometer (Nanodrop 2000; Thermo). Protein (100 µg) was loaded onto a 10% polyacrylamide gel, and Western blot analysis was performed according to standard methods. Proteins were detected using antibodies raised against MmcO (10), MspA (31) and RNA polymerase (Neoclone) and anti-mouse or anti-rabbit secondary antibody linked to horseradish peroxidase; blots were visualized with ECL Western blot detection kit (Thermo). Bands representing proteins of interest were quantified by calculating the integrated optical density (IOD) using LabWorks software (UVP).

alamarBlue Assay

A microplate alamarBlue assay (MABA) was performed as previously described with some minor modifications (38, 39). Briefly, bacterial strains were grown in HdB medium, filtered through a 5-µm filter disc and diluted in 2x HdB medium to an OD_{600} of 0.05. The assay was performed in 96-well microplates. Solutions of copper sulfate,

ampicillin, or spermine were prepared at various concentrations 4-fold higher than the intended final concentration during the assay. Copper and spermine or ampicillin and spermine were mixed in equal parts in the well prior addition of medium and cells. The maximum volume per well was 160 μl. The assay mixtures containing *M. smegmatis* or *M. tuberculosis* were incubated for ~16 h or 6 to 10 days, respectively, prior addition of 30 μl alamarBlue mix (equal parts of alamarBlue dye solution and 10 % Tween 80). Dye conversion was measured fluorometrically (excitation, 530 nm; emission, 590 nm) using a Synergy HT plate reader in bottom reading mode (Biotek).

RESULTS

Porins are Essential for Copper Acquisition by *M. smegmatis*

Previous studies from our laboratory established porins as key mediators of nutrient acquisition in *M. smegmatis* (30). To investigate if porins are also involved in the acquisition of copper, we examined porin mutants of *M. smegmatis* for copper-related growth defects. For this purpose, *M. smegmatis* wild-type and the well-characterized porin mutants MN01 (Δ*mspA*), ML10 (Δ*mspA* Δ*mspC*) and ML16 (Δ*mspA* Δ*mspC* Δ*mspD*) (30) were grown to stationary phase and then normalized by optical density and spotted onto 7H10 solid medium containing trace amounts or 25 µM copper (Fig. 1A and B). Growth was assessed after 3 days at 37°C. The copper-sensitive *M. smegmatis* mutant ML77 (Δ*mctB*) (15) was included to control for copper susceptibility. No apparent growth defect was observed for the wild-type strain under either condition. In contrast, the porin double mutant ML10 and triple mutant ML16 grew poorly on trace copper medium (Fig. 1A). Single colonies of ML16 grown under similar conditions showed a similar growth defect, although much more pronounced (see Fig. S1 in the supplemental material). The growth defects were alleviated either by expression of *mspA* (Fig. 1A; see Fig. S1 in the supplemental material) or by addition of extra copper to the growth medium (Fig. 1B, see Fig. S1 in the supplemental material). The growth of the Δ*mspA* mutant MN01 (Fig. 1A) and the Δ*mspC* mutant ML02 (see Fig. S1 in the supplemental material) was not impaired on trace copper medium, indicating that deletion of only one porin gene is not sufficient to affect copper uptake and that in *M. smegmatis* copper is also taken up by alternatively expressed porins, such as MspB and MspC.

To confirm that the MspA paralogues MspB and MspC are able to take up copper, we individually expressed the genes for these porins in ML10 using syngeneic plasmids that only differ in the porin gene they express (30). Similar expression levels of these porins were confirmed by Western blot analysis (see Fig. S2 in the supplemental material). The drop assay confirmed that the expression of *mspA*, *mspB* or *mspC* promoted growth of ML10 on trace copper medium. In summary, these results indicate that the porin mutants ML10 and ML16 suffer from an uptake defect for copper ions, which appears to be the primary cause for impaired growth on trace copper medium.

Previous studies indicate that MspD is the least conserved and shows the least pore activity of all four Msp porins in *M. smegmatis* (30). Deletion of *mspD* from ML10, which created ML16, did not further decrease the porin content of ML16 (30), which suggests that *M. smegmatis* is unlikely to utilize MspD for nutrient acquisition. In light of these studies, and because ML10 and ML16 had identical copper-dependent phenotypes in our experiments (Fig. 1), we have no indication that MspD is of any significance for the copper metabolism in any of the *M. smegmatis* strains utilized.

Lack of Porins Protects *M. smegmatis* from Copper Poisoning

Although copper supplementation is beneficial for growth of *M. smegmatis*, excess copper is toxic. Because ML10 and ML16 are deficient in copper uptake, we hypothesized that these porin mutants are more resistant to copper. Indeed, the drop assay on 7H10 plates containing 100 µM copper revealed that the porin mutants ML10 and ML16 are copper-tolerant while the growth of wild-type *M. smegmatis* and the single porin mutants MN01 (*ΔmspA*) (Fig. 1C) and ML02 (Δ*mspC*) (data not shown) was

impaired. As expected, expression of *mspA* in either ML10 (Fig. 1F) or ML16 (Fig. 1C) increased sensitivity to copper. Similar results were obtained for *mspB* and *mspC* expressed in ML10 (Fig. 1F). Taken together, these data demonstrate that the susceptibility of *M. smegmatis* to copper ions is a direct function of the porin content in the outer membrane.

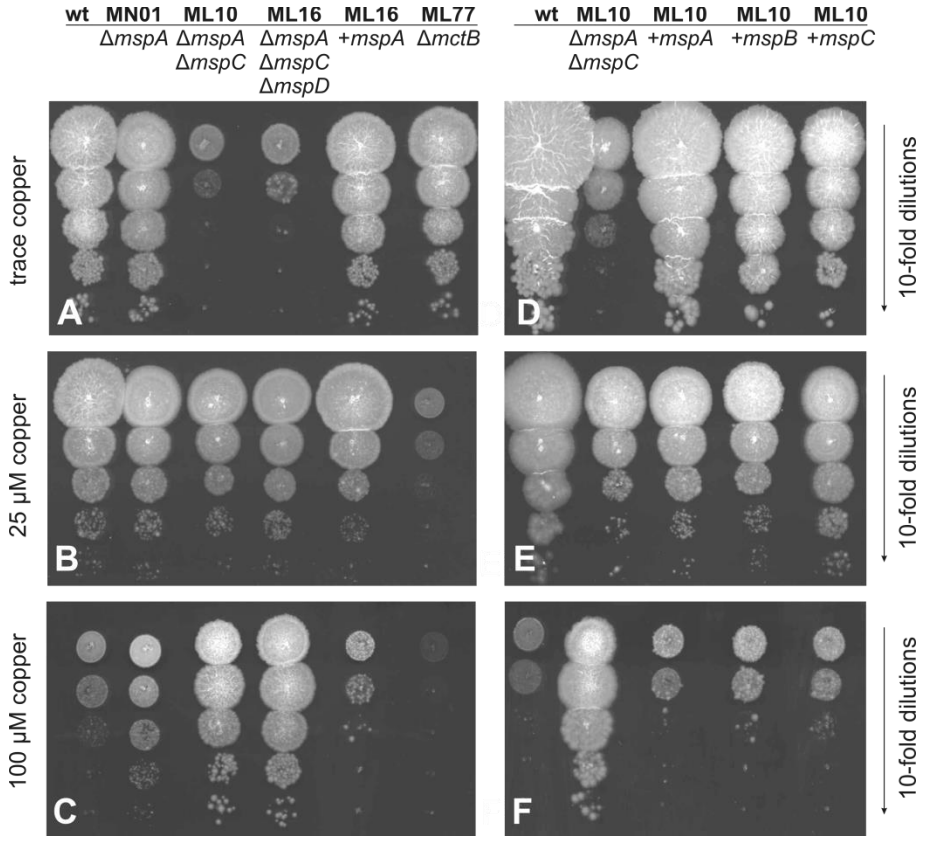

Figure 1. Copper-dependent phenotypes of *M. smegmatis* porin mutants. (A to C) Ten-fold serial dilutions of wild-type *M. smegmatis* SMR5 (wt) and the porin mutants MN01 (Δ*mspA*), ML10 (Δ*mspA* Δ*mspC*), ML16 (Δ*mspA* Δ*mspC* Δ*mspD*) and the *mspA*-expressing ML16 strain were spotted on self-made Middlebrook 7H10 medium plates containing increasing concentrations of copper. ML77 (Δ*mctB*) was included as copper-sensitive growth control. **(D to F)** The porin genes *mspA*, *mspB* or *mspC* were expressed in *M. smegmatis* ML10 from plasmids pMN041, pMN042, and pMN043, respectively. The experiments were repeated four times with similar outcomes. Images of plates containing trace **(A and D)**, 25 µM **(B and E)** or 100 µM **(C and F)** copper from one representative experiment are shown. The copper content of HdB medium was <1 µM.

Growth Kinetics of *M. tuberculosis* in the Presence of Copper

To evaluate at which concentration copper ions affect the growth of *M. tuberculosis*, we utilized the microplate alamarBlue assay which is the current standard for susceptibility testing of mycobacteria (40). Under the applied conditions, we did not detect any growth of *M. tuberculosis* at or above 15 µM copper in HdB medium (Fig. 2A). This outcome accurately reflects our previous finding that *M. tuberculosis* is more sensitive towards copper than *M. smegmatis* (15) and demonstrates suitability of alamarBlue for testing of copper susceptibility in mycobacteria. However, due to the small sample volume, the alamarBlue assay is prone to errors from evaporation and thus not suitable for long-term growth experiments with slow growing mycobacteria. Growth curves over a period of 24 days were therefore recorded by measuring the optical density of 50-ml cultures. In contrast to the copper susceptibility profile of *M. tuberculosis* obtained in alamarBlue assays, we found that *M. tuberculosis* is actually able to grow in the presence of 15 µM copper but growth is delayed and starts on day 10, while growth in trace copper medium began on day 6 (Fig. 2B). The generation time of *M. tuberculosis* increased from ~50 h in trace copper HdB medium to ~75 h in 15 µM copper. Both cultures entered stationary phase at similar cell densities (Fig. 2B). The delayed-growth phenotype in copper medium demonstrates that *M. tuberculosis* has the ability to recover from and adapt to elevated copper levels over time. Indeed, increasing the incubation time of the alamarBlue assay from 7 to 9 days did reveal growth of *M. tuberculosis* at 15 and even at 17.5 µM copper. However, the adaptive copper resistance mechanisms reached their upper limit at ~20 µM copper (see Fig. S3A and B in the supplemental material).

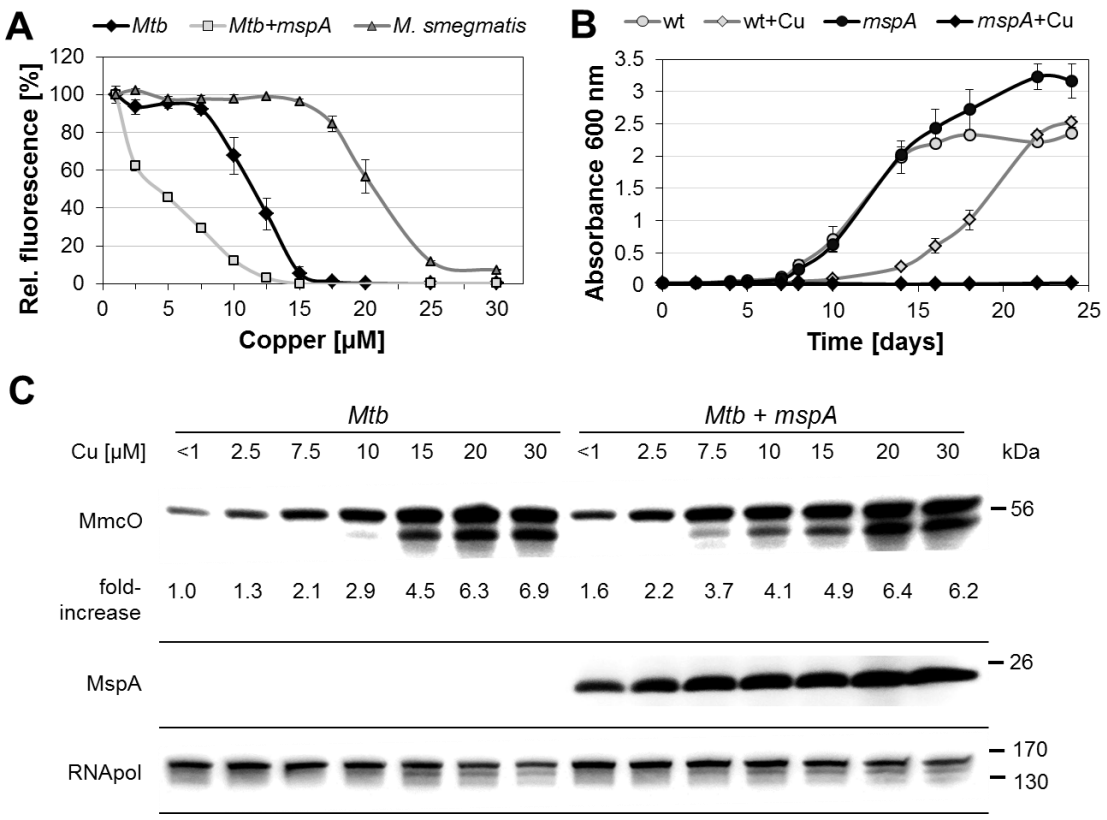

Figure 2. Response of *M. tuberculosis* mc^{2}6230 to copper. (A) alamarBlue assay of *M. tuberculosis* mc^{2}6230 (*Mtb*), *Mtb* expressing *mspA* (*Mtb + mspA*) and *M. smegmatis* in response to increasing copper concentrations. The error bars represent standard deviations of three replicate wells (in a 96-well plate) of the same experiment. **(B)** Growth curves of *M. tuberculosis* mc^{2}6230 (grey lines) and *M. tuberculosis* mc^{2}6230 expressing *mspA* (black lines) in HdB minimal medium containing 15 µM (diamonds) or trace amounts (circles) of copper. Growth was monitored by measuring light scattering at 600 nm (OD_{600}). Standard deviations were calculated from samples of three 50-ml cultures grown in parallel. **(C)** Western blot analysis of MmcO, MspA and RNA polymerase (RNApol) from protein extracts of *M. tuberculosis* mc^{2}6230 and *M. tuberculosis* mc^{2}6230 expressing *mspA* grown at the indicated copper concentrations. The band intensities of MmcO were quantified and normalized to the respective RNA polymerase signals, which were also used as a loading control. Then the samples were compared with wild-type *M. tuberculosis* mc^{2}6230 grown in trace copper medium.

Overexpression of Porin Genes Induces Copper Stress in *M. tuberculosis*

To characterize the role of the outer membrane in protection against copper stress, we expressed the *M. smegmatis* porin *mspA* in *M. tuberculosis* to promote copper uptake across the outer membrane. In contrast to wild-type *M. tuberculosis*, the presence of only 2.5 µM copper inhibited the growth of the *mspA*-expressing strain by ~40% (Fig. 2A). Importantly, while wild-type grew normally at 15 µM copper, the *mspA*-expressing strain did not resume growth during the entire observation period of 24 days (Fig. 2B). This result indicates that the MspA-mediated increase in copper uptake is detrimental to *M. tuberculosis* and highlights the importance of the outer membrane as a diffusion barrier against copper. In addition, we found that expression of *mspA* lead to a stronger copper-dependent induction of multicopper oxidase gene *rv0846c* (*mmcO*) which is part of the *M. tuberculosis* RicR regulon (20). In trace copper medium, the baseline protein level of MmcO in the *mspA* expressing *M. tuberculosis* strain was 60% above wild-type level (Fig. 2C). At 2.5 and 7.5 µM copper, expression levels of MmcO increased to 70% and 76% above wild-type level, respectively (Fig. 2C). These results indicate that copper ions entering the cells through MspA end up in the cytoplasm, where they bind RicR, which results in the transcriptional derepression of copper resistance genes, including *mmcO*.

Spermine Decreases MspA-Mediated Susceptibility to Ampicillin and Copper

Spermine and other polyamines are known modulators of bacterial porin functions (41-43). Thus, we hypothesized that porin-mediated uptake of copper could be blocked by the addition of spermine. To establish this assay, we first demonstrated the ability of

spermine to block uptake of ampicillin through porins of *M. smegmatis*. Ampicillin is an ideal substrate to investigate the inhibitory activity of spermine on MspA for two reasons: (i) ampicillin has been demonstrated to mainly utilize Msp porins to access the periplasm of *M. smegmatis* (44) and (ii) *β*-lactam antibiotics were previously used to detail the interaction between spermine and porins of *E. coli* and *P. aeruginosa* (45, 46). The latter studies demonstrated that spermine increased resistance to *β*-lactam antibiotics by decreasing porin-mediated outer membrane permeability, which also resulted in specific susceptibility phenotypes. To examine if spermine would act in a similar manner on mycobacterial porins, we first tested its capacity to desensitize *M. smegmatis* to ampicillin. alamarBlue assays revealed that spermine indeed enhanced growth of *M. smegmatis* in the presence of 80 µg/mL ampicillin in a spermine concentration-dependent manner (Fig. 3A). In the absence of any toxic compound, spermine had no growth-promoting properties. Further, spermine at 25 µM and 50 µM desensitized *M. smegmatis* to copper (Fig. 3B). Similarly, spermine increased the resistance of *M. tuberculosis* expressing the porin gene *mspA* to copper concentrations of 2.5 and 5 µM (Fig. 3C) but had only minor effects on wild-type *M. tuberculosis* growing at these copper concentrations (see Fig. S3 in the supplemental material). These results indicate that spermine partially blocks the MspA channel in a dose-dependent manner and substantiated our hypothesis that copper enters mycobacterial cells through outer membrane pores.

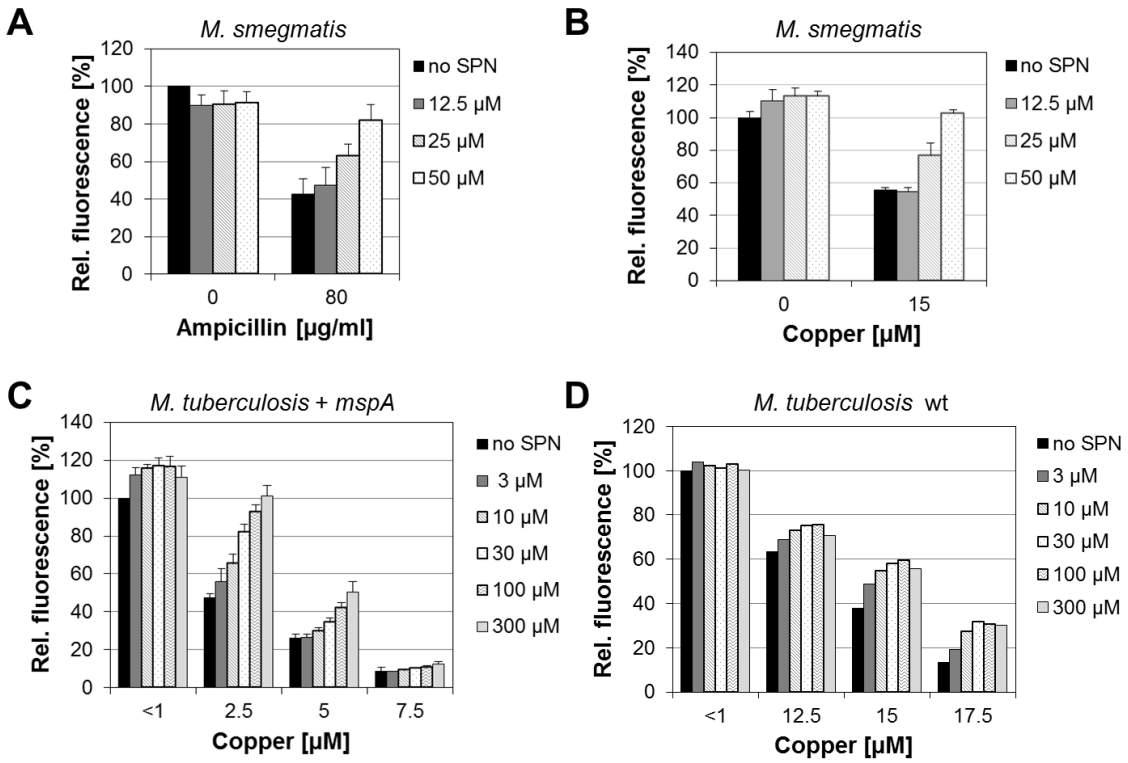

Figure 3. Spermine increases the resistance of *M. smegmatis* and *M. tuberculosis* mc^{2}6230 to ampicillin and copper ions. *M. smegmatis* (A and B), *M. tuberculosis* expressing *mspA* (C) and *M. tuberculosis* (D) were exposed to either ampicillin (A) or copper (B, C and D) in combination with varying concentrations of the polyamine spermine (SPN). Plot D is a partial representation of Fig. S3A in the supplemental material. The error bars indicate the standard deviation of at least 3 technical replicates. The assays were repeated at least twice and gave similar results.

Evidence for Endogenous Porin-Mediated Copper Uptake in *M. tuberculosis*.

To investigate whether copper uptake in *M. tuberculosis* is also modulated by spermine, we grew *M. tuberculosis* at different combinations of copper (<1 to 20 µM) and spermine (0 to 300 µM) concentrations and utilized alamarBlue to obtain a quantitative readout for growth. The assay revealed that spermine acted in a concentration-dependent manner, showing the strongest protective effect in the presence

of 15 and 17.5 µM copper (Fig. 3D; see Fig. S3 in the supplemental material). To better demonstrate the growth-enhancing effect of spermine in the presence of copper, we calculated the spermine-dependent increase of growth, for trace amounts and 12.5, 15 and 17.5 µM copper (see Fig. S4 in the supplemental material) using the respective alamarBlue fluorescence values shown in Fig. S3A in the supplemental material. Spermine did not enhance growth at or below 12.5 µM copper (see Fig. S3 and S4 in the supplemental material), which was to be expected, as these copper concentrations are not inhibitory to *M. tuberculosis* (Fig. 2A). Spermine was most effective in the presence of 17.5 µM copper and enhanced the growth of *M. tuberculosis* by ~2.3 fold (see Fig. S4 in the supplemental material). Similar trends were observed in three independently performed assays (see Fig. S3 in the supplemental material). As spermine is known to block porins (43, 45, 46), and as we demonstrate in this study that spermine blocks the mycobacterial porin MspA, these results indicate that copper uptake by *M. tuberculosis* is a porin-driven process.

DISCUSSION

Msp Porins Mediate Copper Uptake in *M. smegmatis*

Copper acquisition by eukaryotic cells is well characterized (47, 48), but little is known about copper uptake in bacteria. In this study, we observed two distinct copper-dependent phenotypes of *M. smegmatis* porin mutants: (i) a growth defect on trace copper medium (Fig. 1A; see Fig. S1 in the supplemental material) and (ii) increased resistance to elevated copper levels (Fig. 1C; see Fig. S1 in the supplemental material). Both phenotypes indicate that porins are the main mediators for copper uptake across the outer membrane in *M. smegmatis*.

Of all the essential trace metals ions, only uncomplexed iron is known to utilize the porin MspA, which was demonstrated by decreased accumulation of radioactive ^{55}Fe by the *M. smegmatis* triple porin mutant ML16 (49). Porins typically discriminate substrates based on molecular size, type of charge, and/or hydrophobicity (50, 51). Based on these parameters, iron and copper ions appear nearly indistinguishable. Thus, it is likely that, as seen for iron, copper uses Msp porins to enter cells of *M. smegmatis*. Despite our long-standing experience with radioactive uptake assays in mycobacteria (30, 37, 44, 49, 52, 53), we were unable to obtain reliable uptake data with ^{64}Cu(II) due to its fast decay (half-life ~12.7h) and the high affinity of the mycobacterial cell envelope for copper ions.

Instead, we demonstrated the MspA-dependent increase of copper in the cytoplasm by measuring protein levels of the multi-copper oxidase MmcO in response to copper. Expression of *mmcO* is controlled by RicR, a transcriptional repressor of *M. tuberculosis* that functions as an intracellular copper sensor (20). Copper binding to RicR

alters its conformation, resulting in release from its DNA target in a dose-dependent manner, which then leads to an increase in mRNA levels of RicR-controlled genes (20). We demonstrated this correlation by the rising protein levels of MmcO in response to excess copper (Fig. 2C); as RicR needs to physically interact with copper for transcription derepression to occur (20), higher MmcO levels are a consequence of an increasing intracellular copper concentration. Heterologous expression of *mspA* in *M. tuberculosis* led to an even stronger expression of *mmcO* in comparison to wild-type (Fig. 2C), which supports the notion that MspA mediates influx of copper in mycobacteria. In conclusion, the porin-dependent copper-specific phenotypes that we describe in this study and the MspA-dependent increase of *mmcO* expression in response to copper provide the first experimental evidence that copper uptake across bacterial outer membranes is a porin-mediated process.

Redundancy of Porins in Copper Uptake by *M. smegmatis*.

We demonstrated that copper uptake is a redundant function of Msp porin paralogues, as expression of *mspA*, *mspB* or *mspC* restored the wild-type phenotypes in the *M. smegmatis* double porin mutant ML10 (Fig. 1D to F). Deletion of just one porin gene, e.g., *mspA*, in MN01 (Fig. 1A to C) or *mspC* in ML02 (see Fig. S1 in the supplemental material), was not sufficient to generate copper-specific phenotypes, most likely because deletion of one porin gene leads to increased expression of others (30). Expression of alternative porin genes might also explain why copper-specific phenotypes were not previously observed in single (Δ*ompF* or Δ*ompC*) or double (Δ*ompF* Δ*ompC*) porin mutants of *E. coli* (26). Lack of *ompF* and *ompC* is known to induce expression of

phoE (50), a porin usually expressed under phosphate limiting conditions (54). Despite its label as phosphoporin, diffusion through the PhoE pore is not limited to anions (55) and thus is likely to facilitate diffusion of copper ions in the absence of OmpF and OmpC.

Transcriptional profiling of copper-stressed *P. aeruginosa* cells also indicated that copper uptake is achieved by various porins (56). The expression of at least eight porin genes was down regulated, including *oprC*, a TonB-dependent outer membrane protein with a potential role in copper uptake under anaerobic conditions (56). In support of copper uptake being achieved through multiple porins, copper susceptibility of the *oprC* mutant of *P. aeruginosa* was no different from that of the wild-type (57).

Lack of Msp-like Porins Protects *M. tuberculosis* from Copper Stress

The inability of *M. tuberculosis* to grow in the presence of 15 µM copper when *mspA* is expressed (Fig. 2B) shows that *M. tuberculosis* benefits from the lack of MspA-like porins, especially when copper approaches hazardous concentrations (>10 µM). This finding is relevant, as *M. tuberculosis* is likely exposed to copper concentrations well above 15 µM during the course of infection. For example, copper levels in human blood are usually between 15 and 25 µM (58). Copper levels in macrophage phagosomes, in which *M. tuberculosis* survives and replicates (59), can rise up to ~400 µM (18) and multiple studies indicate that copper resistance mechanisms are crucial for full virulence of *M. tuberculosis* (15, 16). According to our results, the outer membrane is particularly important for copper resistance, as it represents an important barrier against toxic copper ions. When this barrier was permeabilized by heterologous expression of the *M. smegmatis* porin gene *mspA*, *M. tuberculosis* became susceptible to as little as 2.5 µM

copper (Fig. 3A). The sensitivity to such a low copper concentration was surprising, as the protein level of MspA in *M. tuberculosis* is very low and does not exceed ~65 pores per μm^2 outer membrane compared to ~1,500 pores per μm^2 in wild-type *M. smegmatis* (37). Supposing that the copper uptake across the outer membrane is greater the more pores are present, it seems evident that the metabolism of *M. smegmatis* is adjusted to a much higher copper influx than the metabolism of *M. tuberculosis*. The failure of *M. tuberculosis* to grow at 15 µM copper when *mspA* is expressed (Fig. 2B) supports this conclusion, as does our previous finding that the level of cell-associated copper in *M. tuberculosis* is approximately five orders of magnitude lower than that of *M. smegmatis* (15).

Spermine Inhibits Porin-Mediated Susceptibility to Ampicillin and Copper in Mycobacteria.

The inhibitory properties of spermine on MspA are indicated by (i) the spermine-induced resistance of *M. smegmatis* to ampicillin and copper ions and, (ii) the spermine-mediated increase in copper resistance by *M. tuberculosis* when *mspA* is expressed. Together, these phenotypes support the conclusion that spermine interferes with the channel activity of MspA and establish spermine as chemical probe to study the permeability of the mycobacterial outer membrane to hydrophilic solutes.

We also demonstrated that the growth of wild-type *M. tuberculosis* in copper-containing media was enhanced by spermine (Fig. 3D), albeit not as strongly as for the *mspA*-expressing *M. tuberculosis* strain (Fig. 3C). It therefore stands to reason that *M. tuberculosis* acquires copper by porins and that spermine acts on endogenous porins of

M. tuberculosis in a manner similar to that we observed for the *M. smegmatis* porin MspA. The striking similarity of spermine-induced phenotypes between mycobacteria and Gram-negative bacteria also prompted Sarathy *et al.* in their recent study to postulate that in *M. tuberculosis* and *Mycobacterium bovis* BCG, fluoroquinolones are being taken up by porins (60). Although our *M. tuberculosis* spermine assays support their conclusion, it should be noted that not all porins are susceptible to polyamines (41) and that spermine and other polyamines are also known to alter bacterial susceptibility to toxic solutes by mechanisms independent of porins (46). These aspects were seemingly overlooked when the spermine-induced drug resistance phenotype of *M. bovis* BCG and *M. tuberculosis* was attributed solely to the blockage of putative porins without providing any evidence that mycobacterial porin functions are indeed susceptible to inhibition by polyamines (60). A direct link between spermine-induced susceptibility phenotypes of *M. tuberculosis* and endogenous porin activities can only be demonstrated in biochemical experiments, which require purified proteins. Unfortunately, as specific porin genes of *M. tuberculosis* are still unknown, such *in vitro* studies are currently impossible. However, the inhibitory effect of spermine on the MspA-mediated uptake of ampicillin and copper we have described provides a reasonable model to explain the reported spermine-induced fluoroquinolone resistance phenotypes of *M. bovis* BCG and *M. tuberculosis* (60).

Regulation of porin functions by endogenously produced polyamines has previously been proposed for *E. coli* (61) but to our knowledge has not yet been examined in *M. tuberculosis*. Indeed, the genome of *M. tuberculosis* seems to encode a putative spermine synthase (*rv2601*), which might indicate that *M. tuberculosis* uses endogenous spermine to modulate porin-mediated influx across its outer membrane.

Furthermore, the spermine concentration in mammalian cells fluctuates but is typically about ~1 mM (62) and therefore high enough to have an impact on porin-mediated uptake processes in *M. tuberculosis*. The effects of host- or bacterium-produced spermine on survival of *M. tuberculosis* have not been characterized.

Alternative Pathways for Copper Uptake in Mycobacteria

The nonavailability of *M. tuberculosis* porin mutants precludes phenotypic studies that detail the role of porins in copper susceptibility, and the copper/spermine assay alone does not exclude the presence of alternative pathways for copper uptake in *M. tuberculosis*. In methanotrophic bacteria, copper appears to be mainly acquired by a system similar to the siderophore-mediated uptake of iron (43). Binding of copper to the virulence associated siderophore yersiniabactin has been reported recently to promote urinary tract infections by *E. coli* (63). Like most bacteria, mycobacteria, including *M. smegmatis* and *M. tuberculosis*, utilize siderophores for the acquisition of iron which is especially crucial for virulence of *M. tuberculosis* (64). However, although copper has been utilized in the purification process of siderophores from *M. tuberculosis*, there is no evidence of a physiological role in copper acquisition, especially as the copper-siderophore complex appears to be unstable (65).

Implications for Drug Discovery

We identified the outer membrane as an important determinant of copper resistance in *M. tuberculosis*. We showed that intracellular copper resistance mechanisms of *M. tuberculosis* were quickly overwhelmed when copper ions were allowed to cross

the outer membrane of *M. tuberculosis* more efficiently. This study is therefore an important step forward to understand how *M. tuberculosis* protects itself from copper-dependent innate immune functions. Accelerated uptake and intracellular accumulation of copper is detrimental to *M. tuberculosis* and weakens its ability to survive in the host (15). Compounds that chemically induce a copper sensitivity phenotype in *M. tuberculosis* may therefore provide an opportunity to therapeutically enhance host-induced copper-dependent innate immune functions. Driven by this hypothesis, the first proof-of-concept compounds have recently emerged (39). Overwhelming the copper resistance mechanisms of *M. tuberculosis* is thus a viable path to new drugs and more effective antituberculosis chemotherapy.

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REFERENCES

1. **Ducluzeau AL, Ouchane S, Nitschke W.** 2008. The *cbb*3 oxidases are an ancient innovation of the domain bacteria. Mol. Biol. Evol. **25:**1158-1166.

2. **Sassetti CM, Boyd DH, Rubin EJ.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol **48:**77-84.

3. **Battistoni A.** 2003. Role of prokaryotic Cu,Zn superoxide dismutase in pathogenesis. Biochem Soc Trans **31:**1326-1329.

4. **Alexandre G, Zhulin IB.** 2000. Laccases are widespread in bacteria. Trends Biotechnol **18:**41-42.

5. **Piddington DL, Fang FC, Laessig T, Cooper AM, Orme IM, Buchmeier NA.** 2001. Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. Infect Immun **69:**4980-4987.

6. **Das D, Bishayi B.** 2010. Contribution of catalase and superoxide dismutase to the intracellular survival of clinical isolates of *Staphylococcus aureus* in murine macrophages. Indian J Microbiol **50:**375-384.

7. **Vanaporn M, Wand M, Michell SL, Sarkar-Tyson M, Ireland P, Goldman S, Kewcharoenwong C, Rinchai D, Lertmemongkolchai G, Titball RW.** 2011. Superoxide dismutase C is required for intracellular survival and virulence of *Burkholderia pseudomallei*. Microbiology 157:2392-2400.

8. **Achard ME, Tree JJ, Holden JA, Simpfendorfer KR, Wijburg OL, Strugnell RA, Schembri MA, Sweet MJ, Jennings MP, McEwan AG.** 2010. The multi-copper-ion oxidase CueO of *Salmonella enterica* serovar Typhimurium is required for systemic virulence. Infect Immun **78:**2312-2319.

9. **Tree JJ, Kidd SP, Jennings MP, McEwan AG.** 2005. Copper sensitivity of *cueO* mutants of *Escherichia coli* K-12 and the biochemical suppression of this phenotype. Biochem Biophys Res Commun **328:**1205-1210.

10. **Rowland JL, Niederweis M.** 2013. A multicopper oxidase is required for copper resistance in *Mycobacterium tuberculosis*. J Bacteriol.

11. **Huston WM, Jennings MP, McEwan AG.** 2002. The multicopper oxidase of *Pseudomonas aeruginosa* is a ferroxidase with a central role in iron acquisition. Mol Microbiol **45:**1741-1750.

12. **Nies DH, Herzberg M.** 2013. A fresh view of the cell biology of copper in enterobacteria. Mol Microbiol **87:**447-454.

13. **Shafeeq S, Yesilkaya H, Kloosterman TG, Narayanan G, Wandel M, Andrew PW, Kuipers OP, Morrissey JA.** 2011. The cop operon is required for copper homeostasis and contributes to virulence in *Streptococcus pneumoniae*. Mol Microbiol **81:**1255-1270.

14. **Schwan WR, Warrener P, Keunz E, Stover CK, Folger KR.** 2005. Mutations in the *cueA* gene encoding a copper homeostasis P-type ATPase reduce the pathogenicity of *Pseudomonas aeruginosa* in mice. Int J Med Microbiol **295:**237-242.

15. **Wolschendorf F, Ackart D, Shrestha TB, Hascall-Dove L, Nolan S, Lamichhane G, Wang Y, Bossmann SH, Basaraba RJ, Niederweis M**. 2011. Copper resistance is essential for virulence of *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 108:1621-1626.

16. **Ward SK, Abomoelak B, Hoye EA, Steinberg H, Talaat AM.** 2010. CtpV: a putative copper exporter required for full virulence of *Mycobacterium tuberculosis*. Mol Microbiol **77:**1096-1110.

17. **White C, Lee J, Kambe T, Fritsche K, Petris MJ.** 2009. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem **284:**33949-33956.

18. **Wagner D, Maser J, Lai B, Cai Z, Barry CE, 3rd, Honer Zu Bentrup K, Russell DG, Bermudez LE.** 2005. Elemental analysis of *Mycobacterium avium*-, *Mycobacterium tuberculosis*-, and *Mycobacterium smegmatis*-containing phagosomes indicates pathogen-induced microenvironments within the host cell's endosomal system. J Immunol **174:**1491-1500.

19. **Liu T, Ramesh A, Ma Z, Ward SK, Zhang L, George GN, Talaat AM, Sacchettini JC, Giedroc DP.** 2007. CsoR is a novel *Mycobacterium tuberculosis* copper-sensing transcriptional regulator. Nat Chem Biol **3:**60-68.

20. **Festa RA, Jones MB, Butler-Wu S, Sinsimer D, Gerads R, Bishai WR, Peterson SN, Darwin KH.** 2011. A novel copper-responsive regulon in *Mycobacterium tuberculosis*. Mol Microbiol **79:**133-148.

21. **Gold B, Deng H, Bryk R, Vargas D, Eliezer D, Roberts J, Jiang X, Nathan C.** 2008. Identification of a copper-binding metallothionein in pathogenic mycobacteria. Nat Chem Biol **4:**609-616.

22. **Rowland JL, Niederweis M.** 2012. Resistance mechanisms of *Mycobacterium tuberculosis* against phagosomal copper overload. Tuberculosis (Edinb) **92:**202-210.

23. **Samanovic MI, Ding C, Thiele DJ, Darwin KH.** 2012. Copper in microbial pathogenesis: meddling with the metal. Cell Host Microbe **11:**106-115.

24. **Rademacher C, Masepohl B.** 2012. Copper-responsive gene regulation in bacteria. Microbiology **158:**2451-2464.

25. **Lutkenhaus JF.** 1977. Role of a major outer membrane protein in *Escherichia coli*. J Bacteriol **131:**631-637.

26. **Li XZ, Nikaido H, Williams KE.** 1997. Silver-resistant mutants of *Escherichia coli* display active efflux of Ag+ and are deficient in porins. J Bacteriol **179:**6127-6132.

27. **Egler M, Grosse C, Grass G, Nies DH.** 2005. Role of the extracytoplasmic function protein family sigma factor RpoE in metal resistance of *Escherichia coli*. J Bacteriol **187:**2297-2307.

28. **Niederweis M.** 2008. Nutrient acquisition by mycobacteria. Microbiology **154:**679-692.

29. **Faller M, Niederweis M, Schulz GE.** 2004. The structure of a mycobacterial outer-membrane channel. Science **303:**1189-1192.

30. **Stephan J, Bender J, Wolschendorf F, Hoffmann C, Roth E, Mailander C, Engelhardt H, Niederweis M.** 2005. The growth rate of *Mycobacterium smegmatis* depends on sufficient porin-mediated influx of nutrients. Mol Microbiol **58:**714-730.

31. **Stahl C, Kubetzko S, Kaps I, Seeber S, Engelhardt H, Niederweis M.** 2001. MspA provides the main hydrophilic pathway through the cell wall of *Mycobacterium smegmatis*. Mol Microbiol **40:**451-464.

32. **Kartmann B, Stenger S, Niederweis M.** 1999. Porins in the cell wall of *Mycobacterium tuberculosis*. J Bacteriol **181:**6543-6546.

33. **Senaratne RH, Mobasheri H, Papavinasasundaram KG, Jenner P, Lea EJ, Draper P.** 1998. Expression of a gene for a porin-like protein of the OmpA family from *Mycobacterium tuberculosis* H37Rv. J Bacteriol **180:**3541-3547.

34. **Sambandamurthy VK, Derrick SC, Hsu T, Chen B, Larsen MH, Jalapathy KV, Chen M, Kim J, Porcelli SA, Chan J, Morris SL, Jacobs WR, Jr.** 2006. *Mycobacterium tuberculosis* DRD1 DpanCD: a safe and limited replicating mutant strain that protects immunocompetent and immunocompromised mice against experimental tuberculosis. Vaccine 24:6309-6320.

35. **Stephan J, Stemmer V, Niederweis M.** 2004. Consecutive gene deletions in *Mycobacterium smegmatis* using the yeast FLP recombinase. Gene **343:**181-190.

36. **Song H, Sandie R, Wang Y, Andrade-Navarro MA, Niederweis M.** 2008. Identification of outer membrane proteins of *Mycobacterium tuberculosis*. Tuberculosis (Edinb) **88:**526-544.

37. **Mailaender C, Reiling N, Engelhardt H, Bossmann S, Ehlers S, Niederweis M.** 2004. The MspA porin promotes growth and increases antibiotic susceptibility of both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. Microbiology **150:**853-864.

38. **Collins L, Franzblau SG.** 1997. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob Agents Chemother **41:**1004-1009.

39. **Speer A, Shrestha TB, Bossmann SH, Basaraba RJ, Harber GJ, Michalek SM, Niederweis M, Kutsch O, Wolschendorf F.** 2013. Copper-boosting compounds: a novel concept for antimycobacterial drug discovery. Antimicrob Agents Chemother **57:**1089-1091.

40. **Stanley SA, Grant SS, Kawate T, Iwase N, Shimizu M, Wivagg C, Silvis M, Kazyanskaya E, Aquadro J, Golas A, Fitzgerald M, Dai H, Zhang L, Hung DT.** 2012. Identification of novel inhibitors of *M. tuberculosis* growth using whole cell based high-throughput screening. ACS Chem Biol 7:1377-1384.

41. **Samartzidou H, Delcour AH.** 1999. Distinct sensitivities of OmpF and PhoE porins to charged modulators. FEBS Lett **444:**65-70.

42. **Iyer R, Delcour AH.** 1997. Complex inhibition of OmpF and OmpC bacterial porins by polyamines. J Biol Chem **272:**18595-18601.

43. **Balasubramanian R, Kenney GE, Rosenzweig AC.** 2011. Dual pathways for copper uptake by methanotrophic bacteria. J Biol Chem **286:**37313-37319.

44. **Danilchanka O, Pavlenok M, Niederweis M.** 2008. Role of porins for uptake of antibiotics by *Mycobacterium smegmatis*. Antimicrob Agents Chemother **52:**3127-3134.

45. **Dela Vega AL, Delcour AH.** 1996. Polyamines decrease *Escherichia coli* outer membrane permeability. J Bacteriol **178:**3715-3721.

46. **Kwon DH, Lu CD.** 2007. Polyamine effects on antibiotic susceptibility in bacteria. Antimicrob Agents Chemother **51:**2070-2077.

47. **Kim BE, Nevitt T, Thiele DJ.** 2008. Mechanisms for copper acquisition, distribution and regulation. Nat Chem Biol **4:**176-185.

48. **Nevitt T, Ohrvik H, Thiele DJ.** 2012. Charting the travels of copper in eukaryotes from yeast to mammals. Biochim Biophys Acta **1823:**1580-1593.

49. **Jones CM, Niederweis M.** 2010. Role of porins in iron uptake by *Mycobacterium smegmatis*. J Bacteriol **192:**6411-6417.

50. **Nikaido H, Rosenberg EY, Foulds J.** 1983. Porin channels in *Escherichia coli*: studies with *b*-lactams in intact cells. J Bacteriol **153:**232-240.

51. **Nikaido H, Rosenberg EY.** 1981. Effect on solute size on diffusion rates through the transmembrane pores of the outer membrane of *Escherichia coli*. J Gen Physiol **77:**121-135.

52. **Song H, Niederweis M.** 2012. Uptake of sulfate but not phosphate by *Mycobacterium tuberculosis* is slower than that for *Mycobacterium smegmatis*. J Bacteriol **194:**956-964.

53. **Wolschendorf F, Mahfoud M, Niederweis M.** 2007. Porins are required for uptake of phosphates by *Mycobacterium smegmatis*. J Bacteriol **189:**2435-2442.

54. **Korteland J, Tommassen J, Lugtenberg B.** 1982. PhoE protein pore of the outer membrane of *Escherichia coli* K12 is a particularly efficient channel for organic and inorganic phosphate. Biochim Biophys Acta **690:**282-289.

55. **Benz R, Schmid A, Hancock RE.** 1985. Ion selectivity of Gram-negative bacterial porins. J Bacteriol **162:**722-727.

56. **Teitzel GM, Geddie A, De Long SK, Kirisits MJ, Whiteley M, Parsek MR.** 2006. Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. J Bacteriol **188:**7242-7256.

57. **Yoneyama H, Nakae T.** 1996. Protein C (OprC) of the outer membrane of *Pseudomonas aeruginosa* is a copper-regulated channel protein. Microbiology **142 (Pt 8):**2137-2144.

58. **Lutsenko S, Barnes NL, Bartee MY, Dmitriev OY.** 2007. Function and regulation of human copper-transporting ATPases. Physiol Rev **87:**1011-1046.

59. **Clemens DL, Horwitz MA.** 1995. Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. J Exp Med **181:**257-270.

60. **Sarathy JP, Lee E, Dartois V.** 2013. Polyamines inhibit porin-mediated fluoroquinolone uptake in mycobacteria. PLoS One **8:**e65806.

61. **Samartzidou H, Delcour AH.** 1999. Excretion of endogenous cadaverine leads to a decrease in porin-mediated outer membrane permeability. J Bacteriol **181:**791-798.

62. **Watanabe S, Kusama-Eguchi K, Kobayashi H, Igarashi K.** 1991. Estimation of polyamine binding to macromolecules and ATP in bovine lymphocytes and rat liver. J Biol Chem **266:**20803-20809.

63. **Chaturvedi KS, Hung CS, Crowley JR, Stapleton AE, Henderson JP.** 2012. The siderophore yersiniabactin binds copper to protect pathogens during infection. Nat Chem Biol **8:**731-736.

64. **Ratledge C.** 2004. Iron, mycobacteria and tuberculosis. Tuberculosis (Edinb) **84:**110-130.

65. **Snow GA.** 1970. Mycobactins: iron-chelating growth factors from mycobacteria. Bacteriol Rev **34:**99-125.

SUPPLEMENTAL FIGURES

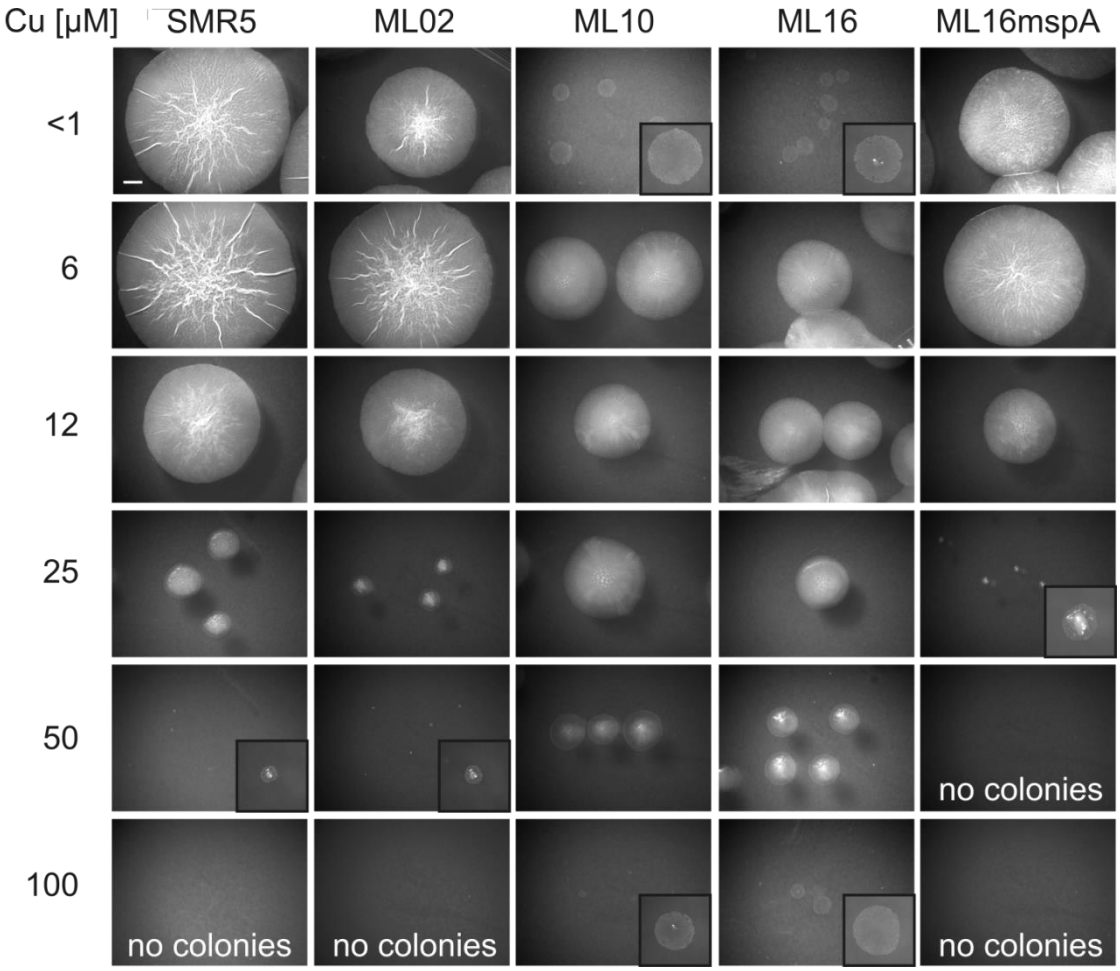

Figure S1. Single colony morphology of *M. smegmatis* SMR5, ML02 (Δ*mspC*), ML10 (Δ*mspA*, Δ*mspC*), ML16 (Δ*mspA*, Δ*mspC*, Δ*mspD)* and ML16 expressing *mspA* grown in the presence of indicated copper concentrations on homemade Middlebrook 7H10 medium plates. A copper concentration of <1 μM indicates trace copper levels. Magnification of colonies is 8-fold. Size bar in top left picture indicates 1 mm at an 8-fold magnification. The insets show colonies at 25-fold magnification.

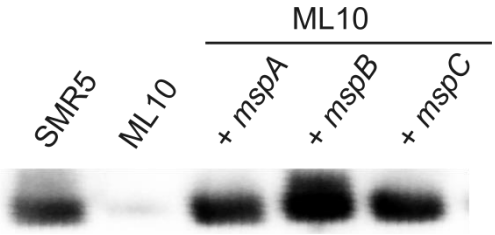

Figure S2. Western blot of porin extracts from *M. smegmatis* SMR5, the porin double mutant ML10 (Δ*mspA*, Δ*mspC*), and ML10 expressing *mspA*, *mspB* or *mspC* from vectors pMN041, pMN042 or pMN043, respectively.

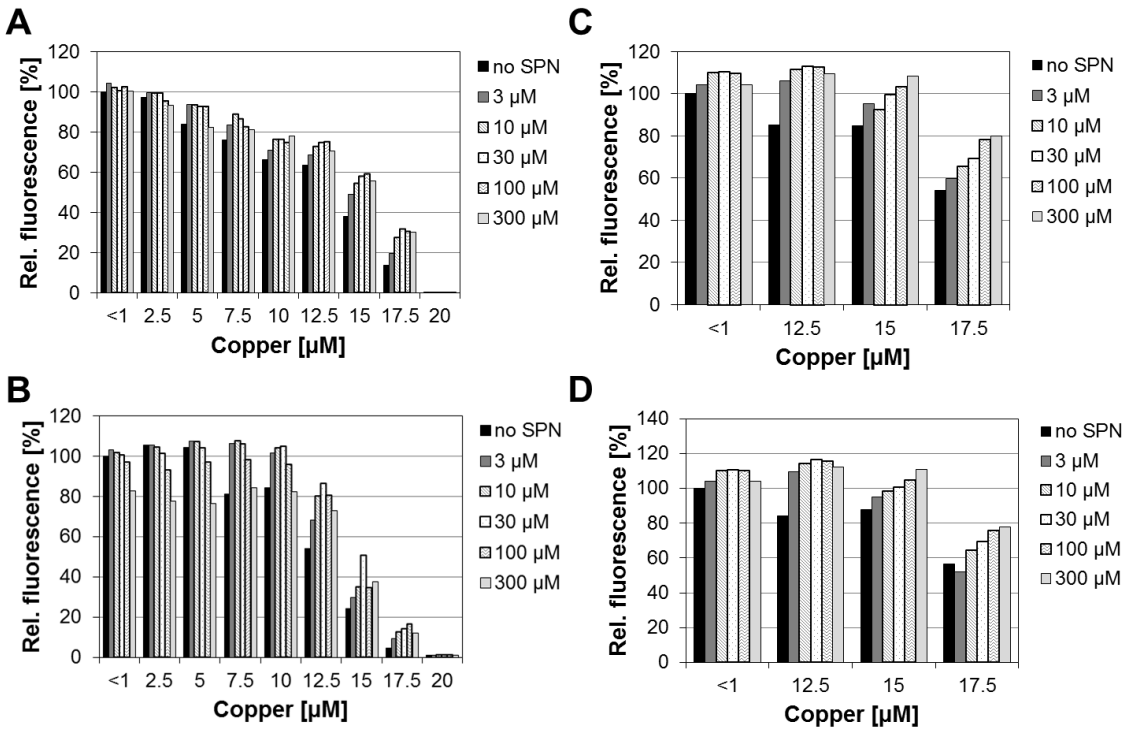

Figure S3. Two-dimensional dose-matrix experiment to demonstrate the growth promoting effects of spermine (SPN) on *M. tuberculosis* $mc^2$6230 in the presence of copper. The results of four independently prepared Alamar Blue assays are shown. The fluorescence value of each condition is normalized to the respective growth control (<1 µM Cu / no SPN). Parts of Fig. S3A are shown in Fig. 3D.

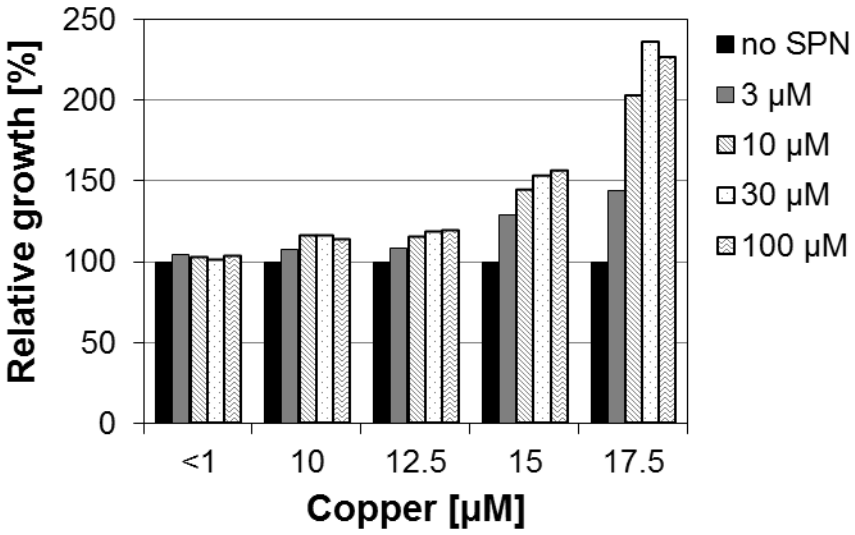

Figure S4. Spermine (SPN) enhances growth of *M. tuberculosis* in the presence of sublethal copper concentrations. Relative growth was calculated from data points shown in Fig. S3A by normalizing relative fluorescence values in each category to the respective no SPN condition.

SUMMARY AND DISCUSSION

MmcO is Critical to Copper Resistance in *M. tuberculosis*

We showed that Rv0846c protein is a *bona fide* multicopper oxidase, and is able to oxidize several organic substrates *in vitro* (Rowland and Niederweis, 2013). Thus we renamed Rv0846c mycobacterial multicopper oxidase (MmcO). In addition to oxidizing organic substrates, we showed the MmcO oxidizes iron. Several multicopper oxidases that oxidize iron also have copper oxidation activity (Singh *et al.*, 2004). We showed that MmcO is required for copper resistance in *M. tuberculosis*, and that activity was dependent on a functional active site, but independent of a putative lipidation site at the amino-terminus (Rowland and Niederweis, 2013). Moreover, strains lacking MmcO activity were more susceptible to copper than previously defined copper-susceptible mutants, such as the Δ*mctB* mutant (Rowland and Niederweis, 2013; Wolschendorf *et al.*, 2011).

The mechanism by which MmcO protects *M. tuberculosis* against copper stress is unclear. The multicopper oxidase of *E. coli*, CueO, is able to oxidize copper directly (Singh *et al.*, 2004). Thus, oxidation of toxic Cu^{1+} to less toxic Cu^{2+} is one possible mechanism of protection by MmcO. Alternatively, CueO can also oxidize iron-loaded siderophores (Kim *et al.*, 2001). It has been hypothesized that siderophores reduce Cu^{2+} to Cu^{1+}, and that CueO oxidation of siderophores reverses this activity (Kim *et al.*, 2001;

Rensing and Grass, 2003). To date, the exact mechanism of multicopper oxidase protection against copper overload has not been shown in any bacterium.

In vivo mechanisms of copper protection may prove difficult to parse. Multicopper oxidases often have low substrate specificity *in vitro* and act on a wide variety of substrates of varying sizes (Kosman, 2010). Indeed, fungal multicopper oxidases can oxidize large, polymeric structures such as lignin (Giardina *et al.*, 2010). Additionally, multicopper oxidases have a variety of roles in different cell types. For example, the circulating multicopper oxidase in human serum, ceruloplasmin, is required for iron delivery to cells and copper and iron homeostasis (Hellman and Gitlin, 2002). Other oxidases, including Fet3P and multicopper oxidase of *Pseudomonas aeruginosa*, are also required for iron homeostasis (De Silva *et al.*, 1995; Huston *et al.*, 2002). The spore coat-associated multicopper oxidase of *Bacillus subtilis* is required for melanin generation, and used in protecting the spore from UV damage (Hullo *et al.*, 2001). For intracellular multicopper oxidases, it is unclear if low substrate specificity is maintained *in vivo*, or if there are mechanisms for limiting activity (Quintanar *et al.*, 2007).

If MmcO acts on small molecule targets *in vivo*, such activities could be identified through a high-throughput mass-spectrometry approach. If MmcO oxidizes small molecules such as siderophores, a comparative lipidomics approach using wildtype and Δ*mmcO* strains would reveal a decrease in the presence of the oxidized form of siderophore in the mutant strain. This approach would also reveal multiple targets of oxidation if they exist.

To date, it has not been shown that a bacterial multicopper oxidase oxidizes copper *in vivo*. All experiments showing cuprous oxidase activity have been performed *in*

vitro, using caged Cu^{1+} substrates under anaerobic conditions (Singh *et al.*, 2004). Methods to show metal oxidation or reduction *in vivo* are limited. Dyes and fluorescent substrates, which change color or fluorescence intensity upon metal binding, are usually not specific, or are not active in cells (Satriano *et al.*, 2013). Metal binding probes are often capable of binding many metals with the same charge and relatively similar size. The *in vitro* copper-sensing dye dithizone is capable of binding multiple metals, including biologically relevant zinc; furthermore, dithizone cannot distinguish the different oxidative states of copper (Paradkar and Williams, 1994). The resulting absorbance spectra of different metal-dithizone complexes overlap precluding deconvolution of different metal or ion-complexes (Paradkar and Williams, 1994). The fluorescent intracellular zinc probe, TPEN, is often described as specific, but in fact binds many biologically relevant metals with higher specificity than zinc (Sigdel *et al.*, 2006).

To avoid convoluted absorbance or excitation-emission spectra, some groups have developed ratiometric metal ion sensors, based on known metal binding proteins. Liu and colleagues engineered a fusion protein of the metal binding domain of a copper-responsive transcriptional regulator, Ace1, and a yellow fluorescent protein, which is specific for Cu^{1+} (Liu *et al.*, 2013). The resulting plasmid-encoded fusion successfully detected changes in the copper pool of bacterial and eukaryotic cells (Liu *et al.*, 2013). Such a fusion protein could be modified with a secretion signal (TAT or Sec) for export to the periplasm, to detect changes in the copper pool of the Δ*mmcO* mutant.

Porins are the Primary Pathway for Copper Uptake in *M. tuberculosis*

The method by which copper enters mycobacterial cells is unknown. In other bacteria with outer membranes, it is assumed that copper utilizes porins, water filled outer membrane channels, to gain access to cells (Nies and Herzberg, 2013). In *E. coli*, it was shown that strains resistant to copper had mutations in genes encoding outer membrane proteins (Lutkenhaus, 1977), but these results were contradicted by later work. Deletion mutants of porins *ompC* and *ompF* were not more resistant to copper than their parent strains, and were even more susceptible to increased copper (Egler *et al.*, 2005; Li *et al.*, 1997). Other bacteria, particularly methanobacteria, use small molecule chelators, called methanobactin (or chalkophores), to bind and accumulate copper (Balasubramanian and Rosenzweig, 2008). This system of copper acquisition is analogous to the siderophore system of iron uptake. Because the mechanism of copper uptake is unknown in mycobacteria, and because copper resistance is critical for virulence, we attempted to characterize copper uptake in *M. smegmatis* and *M. tuberculosis*. There is no evidence for a methanobactin-like system in mycobacteria. *M. smegmatis* has several known porins including MspA (Stephan *et al.*, 2005). *M. tuberculosis* also contains porins, although their identities are as yet unknown (Kartmann *et al.*, 1999). Therefore, we hypothesized that porins are required for copper uptake in mycobacteria.

The *M. smegmatis* genome encodes a well characterized porin, MspA, which has three homologues named MspB, MspC, and MspD. These four porins differ only slightly in amino acid sequence (2, 4, and 18 residues respectively), compared to MspA (Stahl *et al.*, 2001). Specific identities of general porins of *M. tuberculosis* are currently unknown

and there are no homologues of Msp proteins. We, therefore, first characterized the role of general porins in copper susceptibility and resistance in *M. smegmatis*.

We showed that loss of porins results in reduced growth on low copper medium, likely due to limitation of this essential nutrient, and resistance to copper at high concentrations (Speer *et al.*, 2013a). Loss of a single Msp protein resulted in a more subtle copper susceptibility/resistance profile, while loss of two or three Msp proteins (Δ*mspA*, Δ*mspC*; or Δ*mspA*, Δ*mspC*, Δ*mspD*) magnified the phenotype (Speer *et al.*, 2013a). Additionally, the double-porin mutant could be complemented by expression of *mspA*, *mspB*, or *mspC* (Speer *et al.*, 2013a). This result further highlights the dual role of copper in cells, and reveals the importance of balancing copper needs against toxicity.

Because porins have not yet been identified in *M. tuberculosis*, we employed two approaches to show that copper enters through pores. First we overexpressed *mspA* in *M. tuberculosis*. The increase of porins in *M. tuberculosis* increased susceptibility to copper (Speer *et al.*, 2013a). Additionally, we showed that this copper reached the cytoplasm by monitoring expression of a copper responsive gene. Protein levels of MmcO are known to increase in response to copper stress (Rowland and Niederweis, 2013), thus we deteremined MmcO levels by Western blot to show that *mspA*-expressing *M. tuberculosis* accumulated copper in the cytoplasm.

Finally we showed that porin-mediated uptake of copper could be blocked in mycobacteria. Polyamines are known to block porins of Gram-negative bacteria (Iyer and Delcour, 1997; Samartzidou and Delcour, 1999), and have previously been used to investigate copper uptake via porins (Balasubramanian *et al.*, 2011). In *M. smegmatis*, addition of the polyamine spermine increased bacterial resistance to copper, while having

no affect on growth by itself. Addition of spermine to *M. tuberculosis* or *mspA*-expressing *M. tuberculosis* also protected cells against copper stress (Speer *et al.*, 2013a). This result showed that porins are at least partially responsible for copper uptake in *M. tuberculosis*.

These results indicate that porins are utilized in mycobacteria to obtain copper. Particularly, we have identified the Msp proteins as a major pathway for copper uptake in *M. smegmatis*. Without knowing the identities of outer membrane porins or channel proteins in *M. tuberculosis*, we cannot yet identify the exact mechanisms of copper uptake. Identification of channel-forming proteins in *M. tuberculosis* will allow us to further characterize the exact role of outer membrane proteins in copper uptake and susceptibility.

Spatial Separation of Copper Resistance Proteins and Functional Redundancy

The copper resistance proteins are localized to each of the subcellular compartments in the *M. tuberculosis* (Figure 1, introduction). The copper metallothionein, MymT directly protects the cytoplasm by sequestering excess copper ions (Gold *et al.*, 2008). CtpV also protects the cytoplasm by exporting copper into the periplasm (Ward *et al.*, 2010). In turn, the periplasm is protected by MmcO likely by oxidizing toxic Cu^{1+} to less toxic Cu^{2+}. However, alternative mechanisms are possible. MctB is also localized to the periplasm and membrane associated (Siroy *et al.*, in preparation); however, the mechanism by which MctB protects *M. tuberculosis* against copper stress is unclear (Rowland and Niederweis, 2012). It is possible that MctB affects outer membrane integrity and thus loss of MctB results in increased permeability to

copper. Finally, unknown proteins must be present to efflux copper out of *M. tuberculosis*, across the outer membrane.

The spatial distribution of copper resistance proteins in *M. tuberculosis* across bacterial compartments reveals potential redundancies in the system. To determine the overlaps in mechanisms, *M. tuberculosis* mutants with deletions of multiple genes involved in copper resistance must be analyzed. For example, deletion of *ctpV* and *mymT* may reveal a severe copper susceptibility phenotype, as the two encoded proteins protect the cytoplasm against copper overload. Similarly, loss of copper efflux combined with loss of MmcO might be particularly detrimental to the periplasm. It is clear that individual copper resistance proteins are not essential, but disruption of several pathways at once may prove lethal.

Copper Resistance Mechanisms of *M. tuberculosis* are Potential Drug Targets

Copper has long been used as an antimicrobial agent. As early as 3000 BC copper was used to sterilize wounds and water (Grass *et al.*, 2011). Many ancient civilizations used copper to treat a variety of diseases (Grass *et al.*, 2011; Hodgkinson and Petris, 2012). Indeed, copper was used to treat infections, including tuberculosis, until the advent of antibiotics (Grass *et al.*, 2011). Given the increase in multi- and extensively-drug resistant *M. tuberculosis* strains, the use of copper to aid anti-tuberculosis treatments is an intriguing possibility.

Recently there has been renewed interest in using copper in tuberculosis chemotherapy. Beginning with the characterization of MctB, it has been suggested that copper homeostasis mechanisms represent novel drug targets in *M. tuberculosis*

(Wolschendorf *et al.*, 2011). The recent identification of new copper homeostasis mechanisms of *M. tuberculosis* reveals potential drug targets. For example, by blocking copper efflux by CtpV, or inhibiting the activity of MctB, *M. tuberculosis* would be forced to accumulate copper. Loss of CtpV or MctB increases copper susceptibility and leads to virulence defects.

Additionally, inhibiting the copper binding capacity of MymT increases the copper susceptibility of *M. tuberculosis* (Gold *et al.*, 2008). While loss of MymT alone does not result in a virulence defect (Gold *et al.*, 2008), it is possible that loss of MymT function in combination with current or future anti-tuberculosis chemotherapies may result in a more potent drug treatment.

Drugs that target the multicopper oxidase, MmcO, may also be highly effective. It is clear that loss of MmcO is deleterious to *M. tuberculosis*, and induces a high level of copper susceptibility (Rowland and Niederweis, 2013). The localization of MmcO in the periplasm (McDonough *et al.*, 2008) is an additional benefit for several reasons. First, drugs targeting MmcO will increase copper stress in the periplasm, where copper exerts negative effects in multiple ways. Second, loss of multicopper oxidases in other bacteria results in an increase of total bacterial copper content (Tree *et al.*, 2005); as such, inhibition of MmcO oxidase activity may result in an increase in periplasmic and/or cytoplasmic copper, increasing the deleterious effects of the drug. Recent work has shown that MmcO is not required for virulence in mice (Shi *et al.*, 2014). However, unlike other animal models and humans, mice do not form hypoxic granulomas (Via *et al.*, 2008). As such, it is possible MmcO plays a role in virulence in humans.

Another method to utilize copper to target *M. tuberculosis* is the use of copper complexing compounds to overwhelm the native copper resistance mechanisms, instead of targeting individual proteins. Recently, diacetylbis(N(4)-methyl-3-thio-semicarbazone) (ATSM), a bis-thiosemicarbazone used in medical imaging and which is known to bind copper, was used as a proof-of-concept compound to increase the bactericidal effects of copper against *M. tuberculosis*. In the absence of copper, ATSM had almost no inhibitory effect on *M. tuberculosis*; however, upon the addition of copper, ATSM was highly effective (Speer *et al.*, 2013b). These results indicate that overloading copper resistance mechanisms of *M. tuberculosis* is a viable method for drug treatment.

Conclusions

In this work we have improved our understanding of copper homeostasis mechanisms of *M. tuberculosis*. More work is needed to characterize the full complement of copper uptake and resistance systems in *M. tuberculosis*. The identities of porins required for uptake as well as those required for copper efflux are unknown. The copper resistance systems of *M. tuberculosis* also appear redundant, but the exact overlaps between proteins and mechanisms are unknown. We have shown that a putative multicopper oxidase has phenoloxidase and ferroxidase activities. Further we have proven its role in copper resistance. We have also demonstrated that copper enters mycobacteria through general porins. These results advance our understanding of the basic physiology of *M. tuberculosis* and provide a strong platform for future investigations into the role of copper resistance in tuberculosis pathogenicity.

GENERAL REFERENCES

Achard, M.E., Tree, J.J., Holden, J.A., Simpfendorfer, K.R., Wijburg, O.L., Strugnell, R.A., Schembri, M.A., Sweet, M.J., Jennings, M.P., and McEwan, A.G. (2010). The multi-copper-ion oxidase CueO of *Salmonella enterica* serovar *Typhimurium* is required for systemic virulence. Infect Immun *78*, 2312-2319.

Bagai, I., Rensing, C., Blackburn, N.J., and McEvoy, M.M. (2008). Direct metal transfer between periplasmic proteins identifies a bacterial copper chaperone. Biochemistry *47*, 11408-11414.

Balasubramanian, R., Kenney, G.E., and Rosenzweig, A.C. (2011). Dual pathways for copper uptake by methanotrophic bacteria. J Biol Chem *286*, 37313-37319.

Balasubramanian, R., and Rosenzweig, A.C. (2007). Structural and mechanistic insights into methane oxidation by particulate methane monooxygenase. Acc Chem Res *40*, 573-580.

Balasubramanian, R., and Rosenzweig, A.C. (2008). Copper methanobactin: a molecule whose time has come. Curr Opin Chem Biol *12*, 245-249.

Blindauer, C.A. (2011). Bacterial metallothioneins: past, present, and questions for the future. J Biol Inorg Chem *16*, 1011-1024.

Botella, H., Peyron, P., Levillain, F., Poincloux, R., Poquet, Y., Brandli, I., Wang, C., Tailleux, L., Tilleul, S., Charriere, G.M., *et al.* (2011). Mycobacterial p(1)-type ATPases mediate resistance to zinc poisoning in human macrophages. Cell Host Microbe *10*, 248-259.

Brandt, L., Feino Cunha, J., Weinreich Olsen, A., Chilima, B., Hirsch, P., Appelberg, R., and Andersen, P. (2002). Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. Infect Immun *70*, 672-678.

Castillo-Duran, C., Fisberg, M., Valenzuela, A., Egana, J.I., and Uauy, R. (1983). Controlled trial of copper supplementation during the recovery from marasmus. Am J Clin Nutr *37*, 898-903.

Changela, A., Chen, K., Xue, Y., Holschen, J., Outten, C.E., O'Halloran, T.V., and Mondragon, A. (2003). Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. Science *301*, 1383-1387.

Chaturvedi, K.S., Hung, C.S., Crowley, J.R., Stapleton, A.E., and Henderson, J.P. (2012). The siderophore yersiniabactin binds copper to protect pathogens during infection. Nat Chem Biol.

Chaturvedi, K.S., Hung, C.S., Giblin, D.E., Urushidani, S., Austin, A.M., Dinauer, M.C., and Henderson, J.P. (2013). Cupric yersiniabactin is a virulence-associated superoxide dismutase mimic. ACS Chem Biol.

Crichton, R.R., and Pierre, J.L. (2001). Old iron, young copper: from Mars to Venus. Biometals *14*, 99-112.

De Silva, D.M., Askwith, C.C., Eide, D., and Kaplan, J. (1995). The FET3 gene product required for high affinity iron transport in yeast is a cell surface ferroxidase. J Biol Chem *270*, 1098-1101.

Dupont, C.L., Grass, G., and Rensing, C. (2011). Copper toxicity and the origin of bacterial resistance--new insights and applications. Metallomics *3*, 1109-1118.

Egler, M., Grosse, C., Grass, G., and Nies, D.H. (2005). Role of the extracytoplasmic function protein family sigma factor RpoE in metal resistance of *Escherichia coli*. J Bacteriol *187*, 2297-2307.

Failla, M.L., and Hopkins, R.G. (1998). Is low copper status immunosuppressive? Nutr Rev *56*, S59-64.

Festa, R.A., Jones, M.B., Butler-Wu, S., Sinsimer, D., Gerads, R., Bishai, W.R., Peterson, S.N., and Darwin, K.H. (2011). A novel copper-responsive regulon in *Mycobacterium tuberculosis*. Mol Microbiol *79*, 133-148.

Flannagan, R.S., Cosio, G., and Grinstein, S. (2009). Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat Rev Microbiol *7*, 355-366.

Francis, M.S., and Thomas, C.J. (1997). Mutants in the CtpA copper transporting P-type ATPase reduce virulence of *Listeria monocytogenes*. Microb Pathog *22*, 67-78.

Fu, Y., Tsui, H.C., Bruce, K.E., Sham, L.T., Higgins, K.A., Lisher, J.P., Kazmierczak, K.M., Maroney, M.J., Dann, C.E., 3rd, Winkler, M.E., *et al.* (2013). A new structural paradigm in copper resistance in *Streptococcus pneumoniae*. Nat Chem Biol *9*, 177-183.

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and Sannia, G. (2010). Laccases: a never-ending story. Cell Mol Life Sci *67*, 369-385.

Gold, B., Deng, H., Bryk, R., Vargas, D., Eliezer, D., Roberts, J., Jiang, X., and Nathan, C. (2008). Identification of a copper-binding metallothionein in pathogenic mycobacteria. Nat Chem Biol *4*, 609-616.

Gonzalez-Guerrero, M., Raimunda, D., Cheng, X., and Arguello, J.M. (2010). Distinct functional roles of homologous Cu+ efflux ATPases in *Pseudomonas aeruginosa*. Mol Microbiol *78*, 1246-1258.

Grass, G., and Rensing, C. (2001). CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. Biochem Biophys Res Commun *286*, 902-908.

Grass, G., Rensing, C., and Solioz, M. (2011). Metallic copper as an antimicrobial surface. Appl Environ Microbiol *77*, 1541-1547.

Hellman, N.E., and Gitlin, J.D. (2002). Ceruloplasmin metabolism and function. Annu Rev Nutr *22*, 439-458.

Hiniker, A., Collet, J.F., and Bardwell, J.C. (2005). Copper stress causes an *in vivo* requirement for the *Escherichia coli* disulfide isomerase DsbC. J Biol Chem *280*, 33785-33791.

Hodgkinson, V., and Petris, M.J. (2012). Copper homeostasis at the host-pathogen interface. J Biol Chem *287*, 13549-13555.

Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J.M., and Engelhardt, H. (2008). Disclosure of the mycobacterial outer membrane: Cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. Proc Natl Acad Sci U S A *105*, 3963-3967.

Hood, M.I., and Skaar, E.P. (2012). Nutritional immunity: transition metals at the pathogen-host interface. Nat Rev Microbiol *10*, 525-537.

Hullo, M.F., Moszer, I., Danchin, A., and Martin-Verstraete, I. (2001). CotA of *Bacillus subtilis* is a copper-dependent laccase. J Bacteriol *183*, 5426-5430.

Huston, W.M., Jennings, M.P., and McEwan, A.G. (2002). The multicopper oxidase of *Pseudomonas aeruginosa* is a ferroxidase with a central role in iron acquisition. Mol Microbiol *45*, 1741-1750.

Iyer, R., and Delcour, A.H. (1997). Complex inhibition of OmpF and OmpC bacterial porins by polyamines. J Biol Chem *272*, 18595-18601.

Kartmann, B., Stenger, S., and Niederweis, M. (1999). Porins in the cell wall of *Mycobacterium tuberculosis*. J Bacteriol *181*, 6543-6546. (Authors' correction appeared in *J. Bacteriol.* 6**181**, 7650).

Kim, C., Lorenz, W.W., Hoopes, J.T., and Dean, J.F. (2001). Oxidation of phenolate siderophores by the multicopper oxidase encoded by the *Escherichia coli yacK* gene. J Bacteriol *183*, 4866-4875.

Koch, R. (1882). The Etiology of Tuberculosis. Berliner Klinischen Wochenshrift *15*, 221-230.

Kosman, D.J. (2010). Multicopper oxidases: a workshop on copper coordination chemistry, electron transfer, and metallophysiology. J Biol Inorg Chem *15*, 15-28.

Koul, A., Arnoult, E., Lounis, N., Guillemont, J., and Andries, K. (2011). The challenge of new drug discovery for tuberculosis. Nature *469*, 483-490.

Li, X.Z., Nikaido, H., and Williams, K.E. (1997). Silver-resistant mutants of *Escherichia coli* display active efflux of Ag+ and are deficient in porins. J Bacteriol *179*, 6127-6132.

Linder, M.C., and Hazegh-Azam, M. (1996). Copper biochemistry and molecular biology. Am J Clin Nutr *63*, 797S-811S.

Liu, J., Karpus, J., Wegner, S.V., Chen, P.R., and He, C. (2013). Genetically encoded copper(I) reporters with improved response for use in imaging. J Am Chem Soc *135*, 3144-3149.

Liu, T., Ramesh, A., Ma, Z., Ward, S.K., Zhang, L., George, G.N., Talaat, A.M., Sacchettini, J.C., and Giedroc, D.P. (2007). CsoR is a novel *Mycobacterium tuberculosis* copper-sensing transcriptional regulator. Nat Chem Biol *3*, 60-68.

Lutkenhaus, J.F. (1977). Role of a major outer membrane protein in *Escherichia coli*. J Bacteriol *131*, 631-637.

MacMicking, J.D., Taylor, G.A., and McKinney, J.D. (2003). Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science *302*, 654-659.

Macomber, L., and Imlay, J.A. (2009). The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. Proc Natl Acad Sci U S A *106*, 8344-8349.

Macomber, L., Rensing, C., and Imlay, J.A. (2007). Intracellular copper does not catalyze the formation of oxidative DNA damage in *Escherichia coli*. J Bacteriol *189*, 1616-1626.

McDevitt, C.A., Ogunniyi, A.D., Valkov, E., Lawrence, M.C., Kobe, B., McEwan, A.G., and Paton, J.C. (2011). A molecular mechanism for bacterial susceptibility to zinc. PLoS Pathog *7*, e1002357.

McDonough, J.A., McCann, J.R., Tekippe, E.M., Silverman, J.S., Rigel, N.W., and Braunstein, M. (2008). Identification of functional Tat signal sequences in *Mycobacterium tuberculosis* proteins. J Bacteriol *190*, 6428-6438.

Munoz, C., Rios, E., Olivos, J., Brunser, O., and Olivares, M. (2007). Iron, copper and immunocompetence. Br J Nutr *98 Suppl 1*, S24-28.

Niederweis, M., Danilchanka, O., Huff, J., Hoffmann, C., and Engelhardt, H. (2010). Mycobacterial outer membranes: in search of proteins. Trends Microbiol *18*, 109-116.

Nies, D.H., and Herzberg, M. (2013). A fresh view of the cell biology of copper in enterobacteria. Mol Microbiol *87*, 447-454.

Osman, D., Waldron, K.J., Denton, H., Taylor, C.M., Grant, A.J., Mastroeni, P., Robinson, N.J., and Cavet, J.S. (2010). Copper homeostasis in *Salmonella* is atypical and copper-CueP is a major periplasmic metal complex. J Biol Chem *285*, 25259-25268.

Outten, C.E., and O'Halloran, T.V. (2001). Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science *292*, 2488-2492.

Outten, F.W., Huffman, D.L., Hale, J.A., and O'Halloran, T.V. (2001). The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. J Biol Chem *276*, 30670-30677.

Outten, F.W., Outten, C.E., Hale, J., and O'Halloran, T.V. (2000). Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, CueR. J Biol Chem *275*, 31024-31029.

Paradkar, R.P., and Williams, R.R. (1994). Micellar colorimetric determination of dithizone metal chelates. Anal Chem *66*, 2152-2756.

Pena, M.M., Lee, J., and Thiele, D.J. (1999). A delicate balance: homeostatic control of copper uptake and distribution. J Nutr *129*, 1251-1260.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem *25*, 1605-1612.

Piddington, D.L., Fang, F.C., Laessig, T., Cooper, A.M., Orme, I.M., and Buchmeier, N.A. (2001). Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. Infect Immun *69*, 4980-4987.

Prohaska, J.R., and Lukasewycz, O.A. (1981). Copper deficiency suppresses the immune response of mice. Science *213*, 559-561.

Purdy, G.E., Niederweis, M., and Russell, D.G. (2009). Decreased outer membrane permeability protects mycobacteria from killing by ubiquitin-derived peptides. Mol Microbiol *73*, 844-857.

Quintanar, L., Stoj, C., Taylor, A.B., Hart, P.J., Kosman, D.J., and Solomon, E.I. (2007). Shall we dance? How a multicopper oxidase chooses its electron transfer partner. Acc Chem Res *40*, 445-452.

Rensing, C., and Grass, G. (2003). *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol Rev *27*, 197-213.

Rodriguez, G.M. (2006). Control of iron metabolism in *Mycobacterium tuberculosis*. Trends Microbiol *14*, 320-327.

Rowland, J.L., and Niederweis, M. (2012). Resistance mechanisms of *Mycobacterium tuberculosis* against phagosomal copper overload. Tuberculosis (Edinb) *92*, 202-210.

Rowland, J.L., and Niederweis, M. (2013). A multicopper oxidase is required for copper resistance in *Mycobacterium tuberculosis*. J Bacteriol *195*, 3724-3733.

Samartzidou, H., and Delcour, A.H. (1999). Distinct sensitivities of OmpF and PhoE porins to charged modulators. FEBS Lett *444*, 65-70.

Satriano, C., Sfrazzetto, G.T., Amato, M.E., Ballistreri, F.P., Copani, A., Giuffrida, M.L., Grasso, G., Pappalardo, A., Rizzarelli, E., Tomaselli, G.A., *et al.* (2013). A ratiometric naphthalimide sensor for live cell imaging of copper(I). Chem Commun (Camb) *49*, 5565-5567.

Schaible, U.E., Sturgill-Koszycki, S., Schlesinger, P.H., and Russell, D.G. (1998). Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium*-containing phagosomes in murine macrophages. J Immunol *160*, 1290-1296.

Shafeeq, S., Yesilkaya, H., Kloosterman, T.G., Narayanan, G., Wandel, M., Andrew, P.W., Kuipers, O.P., and Morrissey, J.A. (2011). The cop operon is required for copper homeostasis and contributes to virulence in *Streptococcus pneumoniae*. Mol Microbiol *81*, 1255-1270.

Shi, L., Sohaskey, C.D., Kana, B.D., Dawes, S., North, R.J., Mizrahi, V., and Gennaro, M.L. (2005). Changes in energy metabolism of *Mycobacterium tuberculosis* in mouse lung and under *in vitro* conditions affecting aerobic respiration. Proc Natl Acad Sci U S A *102*, 15629-15634.

Shi, X., Festa, R.A., Ioerger, T.R., Butler-Wu, S., Sacchettini, J.C., Darwin, K.H., and Samanovic, M.I. (2014). The Copper-Responsive RicR Regulon Contributes to *Mycobacterium tuberculosis* Virulence. MBio *5*.

Sigdel, T.K., Easton, J.A., and Crowder, M.W. (2006). Transcriptional response of *Escherichia coli* to TPEN. J Bacteriol *188*, 6709-6713.

Singh, S.K., Grass, G., Rensing, C., and Montfort, W.R. (2004). Cuprous oxidase activity of CueO from *Escherichia coli*. J Bacteriol *186*, 7815-7817.

Siroy, A., Mailaender, C., Harder, D., Koerber, S., Wolschendorf, F., Danilchanka, O., Wang, Y., Heinz, C., and Niederweis, M. (2008). Rv1698 of *Mycobacterium tuberculosis* represents a new class of channel-forming outer membrane proteins. J Biol Chem *283*, 17827-17837.

Solioz, M., and Stoyanov, J.V. (2003). Copper homeostasis in *Enterococcus hirae*. FEMS Microbiol Rev *27*, 183-195.

Speer, A., Rowland, J.L., Haeili, M., Niederweis, M., and Wolschendorf, F. (2013a). Porins increase copper susceptibility of *Mycobacterium tuberculosis*. J Bacteriol *195*, 5133-5140.

Speer, A., Shrestha, T.B., Bossmann, S.H., Basaraba, R.J., Harber, G.J., Michalek, S.M., Niederweis, M., Kutsch, O., and Wolschendorf, F. (2013b). Copper-boosting compounds: a novel concept for antimycobacterial drug discovery. Antimicrob Agents Chemother *57*, 1089-1091.

Stahl, C., Kubetzko, S., Kaps, I., Seeber, S., Engelhardt, H., and Niederweis, M. (2001). MspA provides the main hydrophilic pathway through the cell wall of *Mycobacterium smegmatis*. Mol Microbiol *40*, 451-464 (Authors' correction appeared in *Mol. Microbiol.* 4**57**, 1509).

Stephan, J., Bender, J., Wolschendorf, F., Hoffmann, C., Roth, E., Mailänder, C., Engelhardt, H., and Niederweis, M. (2005). The growth rate of *Mycobacterium smegmatis* depends on sufficient porin-mediated influx of nutrients. Mol Microbiol *58*, 714-730.

Stoyanov, J.V., Hobman, J.L., and Brown, N.L. (2001). CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. Mol Microbiol *39*, 502-511.

Tree, J.J., Kidd, S.P., Jennings, M.P., and McEwan, A.G. (2005). Copper sensitivity of *cueO* mutants of *Escherichia coli* K-12 and the biochemical suppression of this phenotype. Biochem Biophys Res Commun *328*, 1205-1210.

Via, L.E., Lin, P.L., Ray, S.M., Carrillo, J., Allen, S.S., Eum, S.Y., Taylor, K., Klein, E., Manjunatha, U., Gonzales, J., *et al.* (2008). Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. Infect Immun *76*, 2333-2340.

Wagner, D., Maser, J., Lai, B., Cai, Z., Barry, C.E., 3rd, Honer Zu Bentrup, K., Russell, D.G., and Bermudez, L.E. (2005). Elemental analysis of *Mycobacterium avium*-, *Mycobacterium tuberculosis*-, and *Mycobacterium smegmatis*-containing

phagosomes indicates pathogen-induced microenvironments within the host cell's endosomal system. J Immunol *174*, 1491-1500.

Wang, Y., Zhu, S., Weisman, G.A., Gitlin, J.D., and Petris, M.J. (2012). Conditional knockout of the Menkes disease copper transporter demonstrates its critical role in embryogenesis. PLoS One *7*, e43039.

Ward, S.K., Abomoelak, B., Hoye, E.A., Steinberg, H., and Talaat, A.M. (2010). CtpV: a putative copper exporter required for full virulence of *Mycobacterium tuberculosis*. Mol Microbiol *77*, 1096-1110.

White, C., Lee, J., Kambe, T., Fritsche, K., and Petris, M.J. (2009). A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem *284*, 33949-33956.

Wolschendorf, F., Ackart, D., Shrestha, T.B., Hascall-Dove, L., Nolan, S., Lamichhane, G., Wang, Y., Bossmann, S.H., Basaraba, R.J., and Niederweis, M. (2011). Copper resistance is essential for virulence of *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A *108*, 1621-1626.

WorldHealthOrganization (2013). Global tuberculosis control: WHO report 2012.