Porins Increase Copper Susceptibility of *Mycobacterium tuberculosis*

Alexander Speer,*, Jennifer L. Rowland,*, Mehri Haeili,b,c Michael Niederweis,*, Frank Wolschendorf*b

Department of Microbiology* and Department of Medicine,* University of Alabama at Birmingham, Birmingham, Alabama, USA; Department of Microbiology, School of Biology, College of Science, University of Tehran, Tehran, Iran*

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 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*.

Copper is an important micronutrient and participates in essential metabolic functions in most cells. It is a crucial cofactor 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 cofactors 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 in *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 important not only 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 mycobacteria-specific cytoplasmic copper metallothioneine (MymT) and a periplasmic multicopper oxidase (MmcO) (10, 20, 21). Although copper resistance pathways are of great importance (reviewed in reference 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 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 porins, 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 *Mycobacterium smegmatis* (29, 30). These porins are very similar to each other; MspB, MspC, and MspD diverge in only 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*6230 (ARD1 ΔpanCD) (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*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 (ΔmspA), ML10 (ΔmspA ΔmspC), and ML16 (ΔmspA ΔmspC ΔmspD) has been published previously (30, 31, 35). Control strains carried the empty mycobacterial expression vector pMS2 (36). The pMS2-derived vectors pMN013 (pvinymspA) and pMN016 (pvinymspB) were used for the expression of *mspA* in *M. tuberculosis* and *M. smegmatis*, respectively (30, 37). The isogenic plasmids pMN041, pMN042, and 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 medium 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 overnight in self-made trace copper 7H9 medium. The cultures were filtered through a 5-μm filter disk to remove clumps and then adjusted to an optical density (OD600) of 1 × 10⁻⁶, and 5 μl of each of these serial dilutions was then spotted on self-made 7H10 plates containing trace amounts or 25 or 100 μM copper. The 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 20-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, the cultures were further diluted in 10-fold increments to an OD600 of 1. The OD-adjusted cell suspensions were then incubated 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 20-fold magnification.

**MmcO induction assay.** Strains were grown in 10 ml HdB medium containing only trace amounts of copper ions (<1 μM) (15). At an OD600 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, the cells were harvested by centrifugation for 10 min at 3,000 × 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 × g. The protein content of each sample was determined spectrophotometrically by its A260/A300 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 antimouse or anti-rabbit secondary antibody linked to horseradish peroxidase; blots were visualized with an ECL Western blot detection kit (Thermo). Bands representing proteins of interest were quantified by calculating the integrated optical density (IOD) using LabWorks software (UVp).

**alarBlue 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 2× HdB medium to an OD600 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 to 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 to addition of 30 μl alamarBlue mixture (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, the *M. smegmatis* wild-type and the well-characterized porin mutants MN01 (ΔmspA), ML10 (ΔmspA ΔmspC), and ML16 (ΔmspA ΔmspC ΔmspD) (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 (ΔmcpB) (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 ΔmspA mutant MN01 (Fig. 1A) and the ΔmspC 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 differ only in the porin gene they express (30). Similar expression levels of these porins were confirmed by Western blotting (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 mu-
tants ML10 and ML16 suffer from an uptake defect for copper ions, which appears to be the primary cause of impaired growth on trace copper medium. Previous studies indicated 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 has any significance for 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 the 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) and ML02 (ΔmspC) (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.

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 to copper than M. smegmatis (15) and demonstrates the 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 is 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.
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 the growth of wild-type *M. smegmatis* expressing the porin gene *mspA* to copper (Fig. 3B). Similarly, spermine at 25 μM and 50 μM desensitized *M. smegmatis* to copper (Fig. 3B). Further, 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 *M. tuberculosis* mc26230 expressing high levels of *mmcO* (Fig. 3C). However, the adaptive copper resistance mechanisms reached their upper limit at ~20 μM copper (see Fig. S3A and B in the supplemental material).

**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 the 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* led to stronger copper-dependent induction of the multicopper oxidase gene *rv0846c* (*mmcO*), which is part of the *M. tuberculosis* RicR regulon (20). In the same experiment, the baseline protein level of MmcO in the *mspA*-expressing *M. tuberculosis* strain was 60% above the wild-type level (Fig. 2C). At 2.5 and 7.5 μM copper, expression levels of MmcO increased to 70% and 76% above the 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 the growth of wild-type *M. smegmatis* expressing the porin gene *mspA* 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.

**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 of copper uptake across the outer membrane in *M. smegmatis*. Of all the essential trace metal 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 longstanding 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, ~12.7 h) 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 multicopper 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 the wild type (Fig. 2C), which supports the notion that MspA mediates the 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 (ΔompF or ΔompC) or double (ΔompF ΔompC) porin mutants of E. coli (26).

Lack of mpa and mspC 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 downregulated, including oprC, encoding 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, the 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 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 μm² outer membrane compared to ~1,500 pores per μm² 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 5 orders of magnitude lower than that in 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 a 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 and at higher copper concentrations (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 be demonstrated only 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 ~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 the survival of M. tuberculosis have not been characterized.

Alternative pathways for copper uptake in mycobacteria.

The nonavailability of M. tuberculosis porin variants 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 versiniabactin has been reported recently to promote urinary tract infections by E. coli (63). Like most bacteria, myco-
bacteria, including \textit{M. smegmatis} and \textit{M. tuberculosis}, utilize siderophores for the acquisition of iron, which is especially crucial for virulence of \textit{M. tuberculosis} \cite{44}. Although, however, copper has been utilized in the purification process of siderophores from \textit{M. tuberculosis}, there is no evidence of a physiological role in copper acquisition, especially as the copper-siderophore complex appears unattainable in mammalian cells. \textit{M. tuberculosis} defends the copper-siderophore complex appears to be unattainable (65).

**Implications for drug discovery.** We identified the outer membrane as an important determinant of copper resistance in \textit{M. tuberculosis}. We showed that intracellular copper resistance mechanisms of \textit{M. tuberculosis} were quickly overwhelmed when copper ions were allowed to cross the outer membrane of \textit{M. tuberculosis} more efficiently. This study is therefore an important step forward in understanding how \textit{M. tuberculosis} protects itself from copper-dependent innate immune functions. Accelerated uptake and intracellular accumulation of copper are detrimental to \textit{M. tuberculosis} and weaken its ability to survive in the host (15). Compounds that chemically induce a copper sensitivity phenotype in \textit{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 \cite{39}. Overwhelming the copper resistance mechanisms of \textit{M. tuberculosis} is thus a viable path to new drugs and more effective antituberculosis chemotherapy.

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