RESEARCH ARTICLE

The Copper-Responsive RicR Regulon Contributes to *Mycobacterium tuberculosis* Virulence

Xiaoshan Shi,[a] Richard A. Festa,[a]* Thomas R. Ioerger,[b] Susan Butler-Wu,[a]* James C. Sacchettini,[c] K. Heran Darwin,[a] Marie I. Samanovic[a]

Department of Microbiology, New York University School of Medicine, New York, New York, USA[a]; Department of Computer Science, Texas A&M University, College Station, Texas, USA[b]; Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas, USA[c]

* Present address: Richard A. Festa, Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina, USA; Susan Butler-Wu, Department of Laboratory Medicine Laboratory, University of Washington Medical Center, Seattle, Washington, USA.

ABSTRACT **As with most life on Earth, the transition metal copper (Cu) is essential for the viability of the human pathogen *Mycobacterium tuberculosis*. However, infected hosts can also use Cu to control microbial growth. Several Cu-responsive pathways are present in *M. tuberculosis*, including the regulated in copper repressor (RicR) regulon, which is unique to pathogenic mycobacteria. In this work, we describe the contribution of each RicR-regulated gene to Cu resistance *in vitro* and to virulence in animals. We found that the deletion or disruption of individual RicR-regulated genes had no impact on virulence in mice, although several mutants had Cu hypersensitivity. In contrast, a mutant unable to activate the RicR regulon was not only highly susceptible to Cu but also attenuated in mice. Thus, these data suggest that several genes of the RicR regulon are required simultaneously to combat Cu toxicity *in vivo* or that this regulon is also important for resistance against Cu-independent mechanisms of host defense.**

IMPORTANCE ***Mycobacterium tuberculosis* is the causative agent of tuberculosis, killing millions of people every year. Therefore, understanding the biology of *M. tuberculosis* is crucial for the development of new therapies to treat this devastating disease. Our studies reveal that although host-supplied Cu can suppress bacterial growth, *M. tuberculosis* has a unique pathway, the RicR regulon, to defend against Cu toxicity. These findings suggest that Cu homeostasis pathways in both the host and the pathogen could be exploited for the treatment of tuberculosis.**

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Address correspondence to K. Heran Darwin, heran.darwin@med.nyu.edu, or Marie I. Samanovic, marie.samanovic@nyumc.org.

Mycobacterium tuberculosis is one of the most devastating microbial agents, as it infects nearly one-third of the world's population and kills nearly two million people annually (http://www.who.int/en/). Currently available chemotherapies are lengthy and potentially toxic (1). In addition, the numbers of multiresistant, extensively resistant, and totally drug-resistant strains are rising (2–4). Thus, an improved understanding of *M. tuberculosis* pathogenesis is urgently needed in order to develop improved treatments for tuberculosis.

It has recently been determined that host-derived Cu is important for controlling *M. tuberculosis* infections in two animal models of infection (5). Cu is a well-known antimicrobial agent, but only in the last few years has its role been appreciated with regard to microbial infections in mammals (6). Previous studies found that Cu levels transiently increase in gamma interferon-activated macrophages infected with mycobacteria (7). In another study, it was shown that Cu accumulates within phagolysosomal compartments via the Cu-transporting ATPase ATP7A (8). Additionally, in a guinea pig model of infection, Cu accumulates in the granulomatous lesions of infected lungs (5). Perhaps because of this host response, it appears that *M. tuberculosis* has acquired several independent mechanisms to defend itself against Cu toxicity (6). These include mycobacterial Cu transport protein B (MctB) (5), the Cu-sensitive operon repressor (CsoR) operon (9, 10), and the regulated in Cu repressor (RicR) regulon (11).

The RicR regulon was discovered in an attempt to understand the link between *M. tuberculosis* proteasome function and pathogenesis, as *M. tuberculosis* strains defective for proteasomal degradation are highly attenuated in mice (11–14). This regulon includes *ricR* (encodes a transcriptional repressor), *lpqS* (encodes a putative lipoprotein), *mymT* (encodes a mycobacterial metallothionein), *socAB* (small open reading frame induced by copper A and B), and Rv2963 (a putative permease gene) (11). All five loci are transcriptionally repressed in strains defective for proteolysis by the *M. tuberculosis* proteasome (11). Interestingly, with the exception of *ricR* itself, all of these genes are found only in pathogenic mycobacteria, suggesting that they are important during infections of a vertebrate host. All of these genes have a palindromic motif in their promoters that is recognized by the transcriptional repressor RicR. Like its closely related paralog CsoR, RicR is presumed to bind Cu^{+} and is released from DNA (9, 11). The only previously characterized RicR-regulated gene other than

ricR itself is *mymT*. Although a *mymT* mutant is hypersensitive to Cu, this mutant has no virulence defect in mice (15).

In this study, we sought to determine the contribution of every RicR-regulated gene to Cu resistance and virulence. We found that most of the genes conferred no to variable Cu resistance *in vitro*. Furthermore, none of the single mutants had an attenuated phenotype in mice. In contrast, repression of the entire RicR regulon resulted in a strong Cu-sensitive phenotype *in vitro* and severely attenuated growth *in vivo*. Thus, it appears that multiple members of the RicR regulon are required for Cu resistance during infections.

RESULTS

Most RicR-regulated genes are individually dispensable for Cu resistance *in vitro* and growth in mice. The RicR regulon is presumed to be important for Cu resistance because a *ricR* null mutant, which constitutively expresses all of the genes in the RicR regulon (Fig. 1A), is resistant to high levels of Cu (11). However, the contributions of individual RicR-regulated genes to Cu resistance and virulence had not been determined. Therefore, we sought to quantify the Cu resistance of mutants lacking each RicR-regulated gene. Mutants with three RicR-regulated genes disrupted were identified in our lab collection of more than 10,000 ΦMycoMarT7 mutants in the *M. tuberculosis* H37Rv strain background (16). We also received a previously reported H37Rv *mymT* deletion-disruption strain (15). The genotypes of all of the strains used in this study are described in Table 1.

We used a quantitative liquid-based assay to measure the Cu sensitivity of *lpqS*, Rv2963, *socA*, and *mymT* mutants compared to that of wild-type (WT) *M. tuberculosis* (11). As previously reported, the *mymT* mutant was more sensitive to Cu than WT *M. tuberculosis* or the complemented strain was (Fig. 1B) (15). The *socA* transposon mutant showed WT Cu resistance, while the Rv2963 transposon mutant was slightly (and not always reproducibly) more resistant to Cu (Fig. 1B). Perhaps most interestingly, the *lpqS* transposon mutant was consistently extremely hyperresistant to Cu. We also used a semiquantitative agar plate assay (15, 17) that showed similar Cu susceptibility results for the *mymT* mutant but not the *lpqS* mutant (Fig. 1C).

We next assessed the phenotypes of several mutants in mice. No single mutation attenuated bacterial growth in mice (Fig. 2A). Interestingly, the Rv2963 and *lpqS* mutant bacteria showed increased growth *in vivo* (Fig. 2A). In experiments where we inadvertently used a moderately large inoculum of bacteria (~2,000 CFU/mouse), we unexpectedly observed that mice infected with the *lpqS* mutant were moribund within 4 weeks (Fig. 2B). However, neither gene could restore WT virulence to the respective mutant (data not shown), assuming that the introduction of the WT allele of either gene expressed from its native promoter resulted in appropriate protein synthesis. Thus, it is unclear how (or if) disruption of either gene resulted in the observed hypervirulence phenotypes.

***mmcO* overexpression results in Cu hyperresistance but not hypervirulence.** Upon closer inspection of the *lpqS* locus, we hypothesized that the transposon insertion in the *lpqS* mutant somehow increased the expression of the divergently expressed gene *mmcO* (mycobacterial multicopper oxidase [MCO]) (17) (Fig. 1A and 3A). MCOs exist in all kingdoms of life and play a critical role in Cu and iron homeostasis (18–22). MCOs can oxidize substrates, including reduced metals such as Cu^{+} or Fe^{2+}, as well as phenolic compounds (23–26). Recently it was shown that MmcO can oxidize Fe^{2+} to Fe^{3+} and perhaps can also convert Cu^{+} to Cu^{2+} (17). Additionally, Rowland and Niederweis observed the induction of MmcO production in *M. tuberculosis* upon Cu treatment (17). A microarray analysis determined that *mmcO* is more highly expressed in a *ricR* mutant than in WT *M. tuberculosis*, suggesting that it is Cu and RicR regulated (11). Consistent with these previously published data, we confirmed that MmcO levels are elevated upon Cu treatment and now show that it is also highly abundant in a *ricR* mutant (Fig. 3B) (11).

On the basis of the orientation of the *neo* gene in the ΦMycoMarT7 transposon in *lpqS*, we hypothesized that the *neo* promoter could increase *mmcO* expression in the absence of Cu (Fig. 3A), potentially resulting in the Cu hyperresistance and hypervirulence of this particular strain. We examined MmcO levels in the WT and *lpqS* mutant strains and found that MmcO was more abundant in the *lpqS* mutant than in the WT strain (Fig. 3C).

To determine if MmcO overproduction was responsible for the Cu hyperresistance and hypervirulence phenotypes of the *lpqS* mutant, we deleted and disrupted *mmcO* in this strain. We showed by immunoblotting that MmcO was absent from the double mutant strain (Fig. 4A). A Cu susceptibility assay revealed that deletion of *mmcO* from the *lpqS* mutant restored Cu sensitivity to WT levels, suggesting that MmcO contributed to the Cu hyperresistance of this strain (Fig. 4A). Importantly, the double mutant was no more sensitive to Cu than WT *M. tuberculosis* was, suggesting that *lpqS* itself has a little or no role in Cu resistance. Although Cu hyperresistance was eliminated upon the deletion of *mmcO*, the double mutant was as hypervirulent as the parental *lpqS* strain (Fig. 4B). Thus, MmcO overproduction was not responsible for the hypervirulence of this strain.

We do not understand the nature of the *lpqS* hypervirulence phenotype; complementation with *lpqS* or *lpqS* plus two downstream genes (*cysK2*, Rv0849) could not reduce bacterial growth to WT levels in mice (data not shown). Additionally, whole-genome sequencing of this strain did not reveal any differences from the parental WT strain except for the transposon insertion in *lpqS*.

Deletion of *mmcO* has no effect on virulence in mice. Following the above observation that MmcO can confer Cu hyperresistance when overproduced, we wanted to determine the effect of deleting *mmcO* from WT *M. tuberculosis*. We deleted and disrupted *mmcO* in both the H37Rv and CDC1551 strains. Deletion was confirmed by PCR amplification of the region surrounding the deleted locus (data not shown) and immunoblot detection of MmcO protein (Fig. 5A). Interestingly, when we tested these strains for Cu sensitivity, we found that WT H37Rv was more sensitive to Cu than WT CDC1551 was and we thus had to use higher concentrations of $CuSO_4$ for the CDC1551 strains in our assays. Nonetheless, in both strain backgrounds, the *mmcO* mutants showed WT Cu resistance in our quantitative Cu susceptibility assay (Fig. 5A). However, during the preparation of this report, Rowland and Niederweis showed than an *mmcO* mutant is hypersensitive to Cu by using the semiquantitative agar-based assay (17). We tested our mutants in the agar plate assay and indeed observed that both of our *mmcO* mutants were more sensitive to Cu than the WT or complemented strain was (Fig. 5B).

To determine the importance of *mmcO in vivo*, we infected mice with the H37Rv WT, *mmcO* mutant, and *mmcO*-complemented strains. All of these strains displayed WT growth in

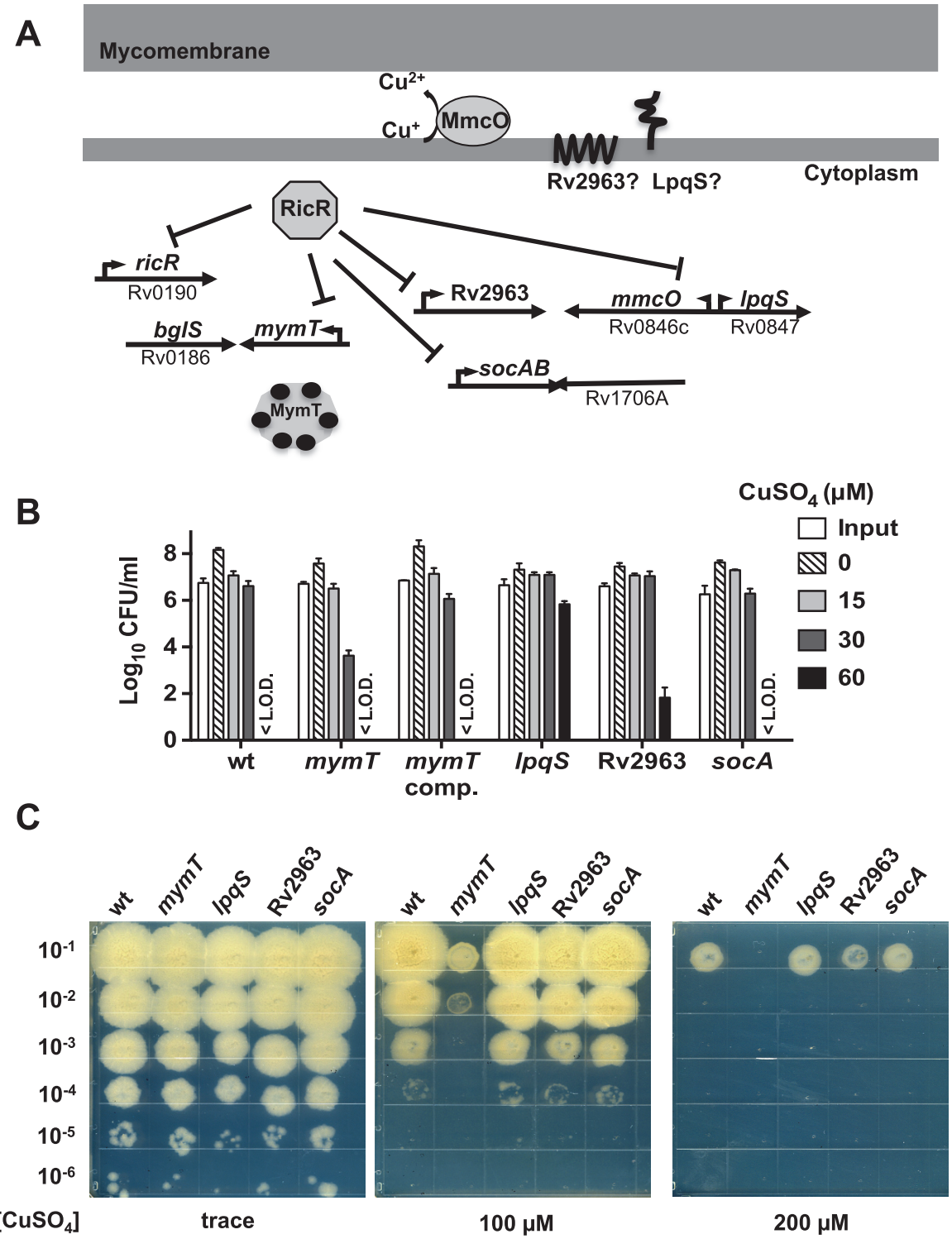

FIG 1 Contribution of RicR-regulated genes to Cu resistance. (A) Model of the RicR regulon in *M. tuberculosis*. Cytoplasmic MymT can bind with up to six Cu^{+} ions (black circles). LpqS and Rv2963 are predicted to be membrane-associated proteins. RicR is autoregulated and also represses *socAB* under low-Cu conditions. MmcO is an MCO. (B) Cu sensitivity assays assessing the ability of RicR regulon mutants to survive at the indicated concentrations of $CuSO_4$ after 10 days. CFU were enumerated after 14 to 21 days of incubation on solid medium with trace amounts of Cu. Data are representative of at least two experiments, each done in triplicate. Abbreviations: comp., complemented; <L.O.D., below the limit of detection (which was 100 CFU). (C) Agar plate assay evaluating the Cu susceptibility of RicR regulon mutants. Serial dilutions of *M. tuberculosis* cultures were spotted onto 7H11-OADC agar plates with the $CuSO_4$ concentrations indicated. The contrast was adjusted to make the images clearer here and in Fig. 5 and 6. Data are representative of two independent experiments.

TABLE 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Phenotype, genotype, or sequence	Source or reference
M. tuberculosis strains		
H37Rv	WT	ATCC 25618
MHD18	Hyg^r WT/pMV306	12
MHD22	Kan^r Hyg^r *mpa*::ΦMycoMarT7/pMV306	12
MHD23	Kan^r Hyg^r *mpa*::ΦMycoMarT7/pMV-*mpa*	12
MHD62	Kan^r Hyg^r *pafA*::ΦMycoMarT7/pMV306	16
MHD63	Kan^r Hyg^r *pafA*::ΦMycoMarT7/pMV-*pafA*	16
MHD131	Kan^r *lpqS*::ΦMycoMarT7 (transposon inserted in codon 44)	This work
MHD188	Kan^r Rv2963::ΦMycoMarT7 (transposon inserted in codon 351)	This work
MHD696	Kan^r *socA*::ΦMycoMarT7 (transposon inserted in codon 22)	This work
MHD701	Hyg^r Δ*mymT*::*hyg*	15
MHD702	Kan^r Hyg^r Δ*mymT*::*hyg*/pMV306.kan-*mymT*	15
MHD752	Hyg^r Δ*mmcO*::*hyg*	This work
MHD764	Kan^r Hyg^r *lpqS*::ΦMycoMarT7 Δ*mmcO*::*hyg*	This work
MHD794	Kan^r WT/pMV306.kan	This work
MHD795	Kan^r Hyg^r Δ*mmcO*::*hyg*/pMV306.kan	This work
MHD796	Kan^r Hyg^r Δ*mmcO*::*hyg*/pMV306.kan-*mmcO*	This work
MHD840	Kan^r Hyg^r Δ*mymT*::*hyg* Δ*mmcO*::*kan*	This work
MHD867	Kan^r Hyg^r Δ*mymT*::*hyg* Δ*mmcO*::*kan*/pMVstrep-*mmcO mymT*	This work
CDC1551	WT	W. Bishai lab collection
MHD557	Kan^r *ricR*::ΦMycoMarT7	14
MHD583	Hyg^r WT/pMV306	11
MHD589	Kan^r Hyg^r *ricR*::ΦMycoMarT7/pMV306	11
MHD590	Kan^r Hyg^r *ricR*::ΦMycoMarT7/pMV-*ricR*	11
MHD694	Kan^r Hyg^r *ricR*::ΦMycoMarT7/pMV-$ricR_{C38A}$	This work
MHD707	Kan^r Hyg^r *ricR*::ΦMycoMarT7/pMV-*ricRp*^{c}-*ricR*	This work
MHD708	Kan^r Hyg^r *ricR*::ΦMycoMarT7/pMV-*ricRp*^{c}-$ricR_{C38A}$	This work
MHD755	Hyg^r Δ*mmcO*::*hyg*	This work
E. coli strains		
DH5α	F$^-$ *ϕ*80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ-*thi-1 gyrA96 relA1*	Gibco BRL
ER2566	F$^-$ λ$^-$ *fhuA2* [*lon*] *ompT lacZ*::T7 gene1 *gal sulA11* Δ(*mcrC-mrr*)*114*::IS*10* R(*mcr-73*::miniTn*10*)*2* R(*zgb-210*::Tn*10*) (Tet^s) *endA1* [*dcm*]	35
Plasmids		
pET24b(+)	Kan^r, for production of C-terminally His_6 epitope-tagged protein	Novagen
pET24b(+)-*mmcO*	Kan^r, for production of MmcO-His_6 in *E. coli*	This work
pET24b(+)-*ricR*	Kan^r, for production of untagged RicR in *E. coli*	11
pET24b(+)-$ricR_{C38A}$	Kan^r, for production of untagged $RicR_{C38A}$ in *E. coli*	This work
pMV306.kan	Kan^r, integrates at *attB* site in mycobacterial chromosome	36
pMV306	Hyg^r, integrates at *attB* site in mycobacterial chromosome	36
pMV306.strep	Strep^r, integrates at *attB* site in mycobacterial chromosome	Gift from J. McKinney lab
pMV306.kan-*mmcO*	Kan^r, for complementation of *mmcO* mutant	This work
pMV-*ricR*	Hyg^r, for complementation of *ricR* mutant	11
pMV-*p*^{c}-*ricR*	Hyg^r, WT *ricR* expressed from mutated *ricRp* (see text)	This work
pMV-*p*^{c}-$ricR_{C38A}$	Hyg^r, $ricR_{C38A}$ allele expressed from mutated *ricRp*	This work
pMVstrep-*mmcO mymT*	Strep^r, for complementation of *mmcO mymT* double mutant	This work
pYUB854	Hyg^r, allelic-exchange vector	32
pYUB854.kan	Kan^r, allelic-exchange vector	This work
pYUB854-*mmcO*	Hyg^r, *M. tuberculosis* Δ*mmcO::hyg* (deletion-disruption plasmid)	This work
pYUB854.kan-*mmcO*	Kan^r, *M. tuberculosis* Δ*mmcO::kan* (deletion-disruption plasmid)	This work
Primers		
NdeI Rv0846c F1	GGCATATGCCCGAGCTGGCCACGAGCGGTAAC	
XhoI Rv0846c R1	GGCTCGAGCAGAATGTAGTCCAGGCGGGTCGC	
Rv0846cF2seq	GGCACCGAGCCCGCGACTGCGAACATC	
Rv0846cR2seq	CCCGGCCAGCGCGATGCGGAACGCGGT	
KpnI 846cMutantF1	GAGGGTACCTATCTGCGGGTTGGAGGTGATGCTTGTTG	
XbaI 846cMutantR1	GTTACCGCTCGTGGCTCTAGACAGCTCGGGCATCGATC	
HindIII 846cMutantF2	ACCCGCCTGGACAAGCTTTACATTCTGTGACAGGCGG	
SpeI 846cMutantR2	TCAGGAGCTCATCGAGTTACTAGTGGATGCCGTAACC	

(Continued on following page)

TABLE 1 (Continued)

Strain, plasmid, or primer	Phenotype, genotype, or sequence	Source or reference
delta*mmcO*F	AGGAGTGACTTGATATCCCTCCGGG	
delta*mmcO*R	ATTGCGGAAGCCATTCACGATGGAC	
HindIII*mmcO*F2	CAAAGCTTACGTGCCCGCTTTCCACGTGGCCC	
*mmcO*compHpaIR	CTGTTAACTCACAGAATGTAGTCCAGGCGGGTC	
C38A SOE F	GTACGCCATTGACGTTCTGACC	
C38A SOE R	GGTCAGAACGTCAATGGCGTAC	
F1*ricR*promsoe	CCGATACCCCGCTGTTGTACAAGATATGAT	
R1*ricR*promsoe	ATCATATCTTGTACAACAGCGGGGTATCGG	
Rv0190 comp F-HindIII	GACAAGCTTCATTGTTCAAGTATGCGGCCCAAG	
Rv0190 comp R-KpnI	GACGGTACCTCAGGAACGAACCAGGCGCGCG	
Rv0190 F NdeI	GACCATATGACAGCAGCACACGGCTACAC	
Rv0190 Rev. Stop EcoRI	GACGAATTCTCAGGAACGAACCAGGCGCGCGATTG	
lpqS affinity F-BIO	BioTEG-ATCGCTCCTCGTCTGGATTT	
lpqS affinity R	AGCGCGACCGCGACAATC	
AgeI pYUBhygtokanfor	CAACCGGTCCCTCCCAAGGACACTGAGTCCTAAAG	
NcoI pYUBhygtokanrev	GTCCATGGTTAGAAAAACTCATCGAGCATCAAATG	
HpaI-mymT-for	GCGGTTAACGGGCGGTTGGGTTGCTGG	
MfeI-mymT-rev	GGCAATTGATAGGTCTACTTGACCGGGGCC	

the lungs and spleens of WT C57BL/6 mice (Fig. 5C). Thus, *mmcO* alone does not significantly contribute to the virulence of *M. tuberculosis* in a mouse model of infection.

Deletion of both *mmcO* and *mymT* is not sufficient to attenuate *M. tuberculosis in vivo*. Deletion of *mmcO* did not result in robust Cu hypersensitivity, suggesting that there might be other Cu-binding proteins that contribute to strong Cu resistance. Because *mymT* is the only other known RicR-regulated gene that significantly contributes to Cu resistance, we deleted and disrupted *mmcO* in the *mymT* deletion-disruption mutant and quantified the Cu resistance and virulence of this double mutant strain. We confirmed that MmcO protein was no longer produced by the double mutant strain (Fig. 6A). In the agar plate assay, the *mmcO mymT* double mutant showed greater Cu susceptibility

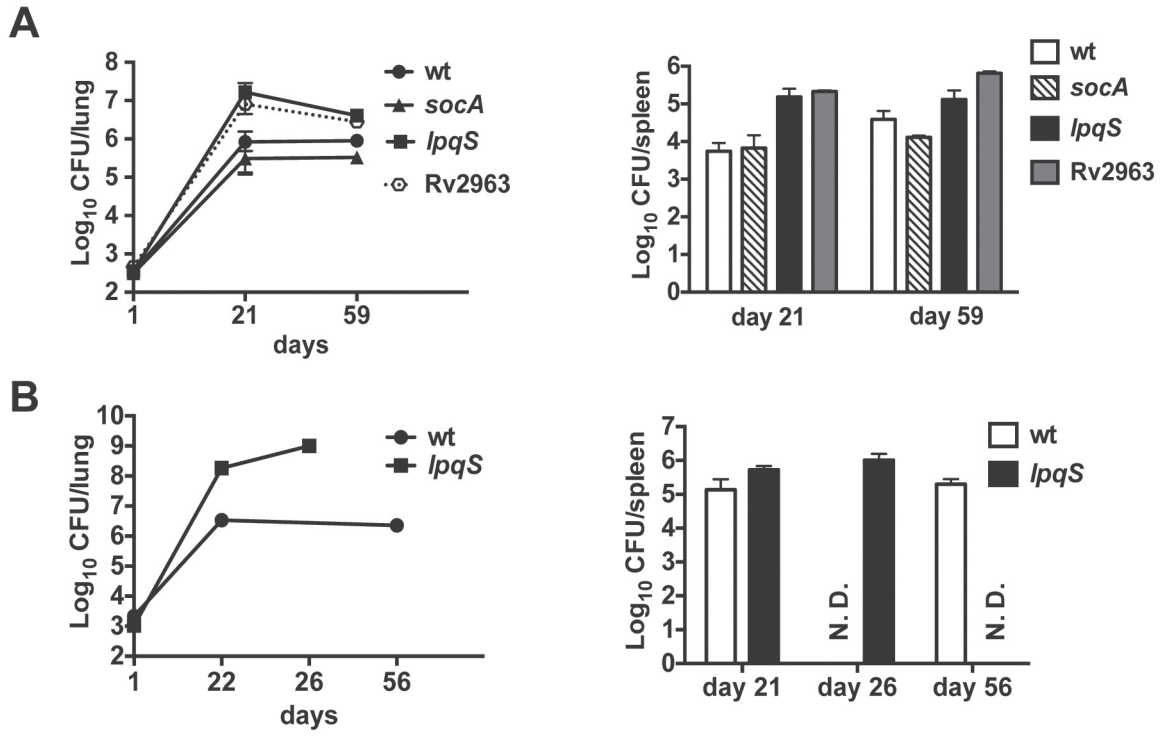

FIG 2 Contribution of RicR-regulated genes to virulence in mice. (A) CFU from lungs and spleens harvested on days 1 ($n = 3$) and 21 ($n = 4$) and at ~8 weeks ($n = 4$) from WT C57BL/6 mice infected with *M. tuberculosis* H37Rv RicR regulon single mutants. The initial dose of infection was ~500 to 1,000 CFU/mouse. Each datum point represents the average number of CFU from organs of three or four mice and the standard deviation. (B) Infection of WT C57BL/6 mice with a moderately large dose (~2,000 CFU/mouse) of WT or *lpqS*::ΦMycoMarT7 (MHD131) *M. tuberculosis*. Because of the slow movement and labored breathing of the mice infected with the *lpqS* mutant, they were sacrificed at day 26. N.D., not determined.

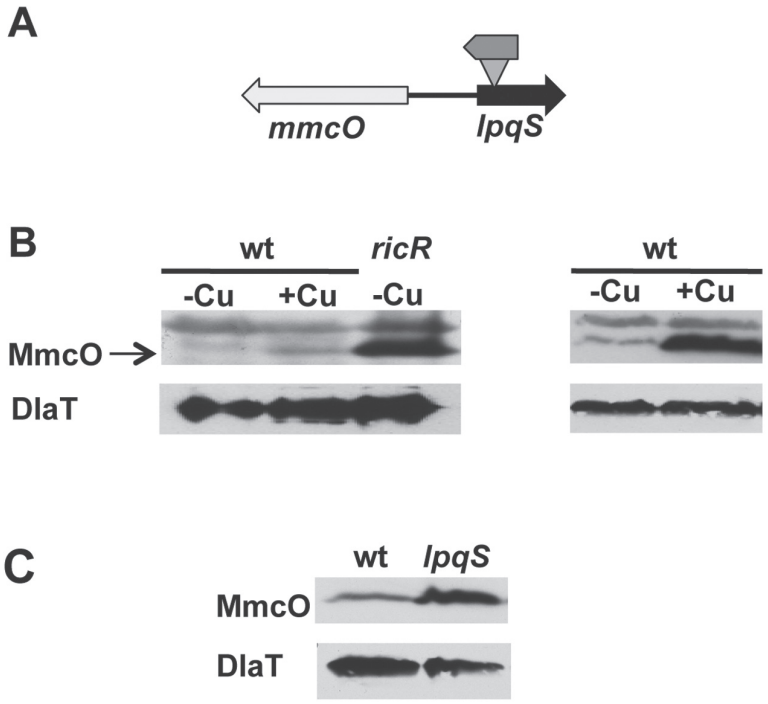

FIG 3 The transposon insertion in MHD131 increased the expression of *mmcO*. (A) Map of the *lpqS* locus in strain MHD131. The ΦMycoMarT7 transposon was inserted at nucleotide (nt) 130 of *lpqS*. *lpqS* is 393 nt long. (B) Immunoblot analysis showed that MmcO increased upon 4 h of Cu treatment or in a *ricR* mutant (left). Data are representative of three biological replicates. In another experiment, robust induction of MmcO was observed after 24 h of treatment with 50 μM $CuSO_4$ (right). (C) MmcO levels were higher in the *lpqS* mutant than in WT *M. tuberculosis*. Immunoblotting for dihydrolipoamide acetyltransferase (DlaT) was used as a loading control for all experiments.

than the *mymT* or *mmcO* single mutant, a phenotype that could be partially complemented with an integrative plasmid encoding *mymT* and *mmcO* expressed from their native promoters (Fig. 6B, top). However, in the liquid-based Cu assay, the double mutant showed Cu sensitivity similar to that of the *mymT* single mutant (Fig. 6B, bottom).

We next infected mice with the WT and single and double mutant strains. None of the mutants demonstrated an attenuated phenotype based on the bacterial burdens found in mouse lungs and spleens up to 8 weeks after aerosol infection (Fig. 6C). Thus, these data suggest that *mmcO* and *mymT* alone are not required for normal replication of *M. tuberculosis* in mice.

Constitutive repression of the RicR regulon results in robust Cu sensitivity *in vitro* and attenuation of *M. tuberculosis* in mice. None of the genes of the RicR regulon seemed individually important for virulence. Therefore, we hypothesized that either several of the RicR-regulated genes are needed or the entire regulon is needed to play a significant role in virulence. Ideally, we would construct an *M. tuberculosis* strain that has all five RicR-regulated loci mutated. Because of the arduous process of deleting multiple genes from *M. tuberculosis*, we developed an alternative method to repress all of the genes in the RicR regulon by producing a "Cu-blind" allele of RicR in a *ricR* null mutant strain. RicR is a homologue of CsoR and has conserved residues that are predicted to bind Cu^+ (9). Cysteine 38 of RicR is predicted to be required for Cu^+ binding (D. Giedroc, personal communication); thus, conversion of cysteine 38 to alanine ($RicR_{C38A}$) would prevent RicR from sensing Cu^+ and detaching from DNA. We previously showed by using a DNA affinity chromatography assay that recombinant RicR binds to a specific sequence *in vitro* under low-Cu conditions and elutes from DNA with increasing amounts of Cu (11) (Fig. 7A, top). In contrast, $RicR_{C38A}$ was unresponsive to Cu and could only be eluted from DNA with a high-salt buffer (Fig. 7A, bottom). Because *ricR* is autoregulated, $RicR_{C38A}$ could also constitutively repress its own production, leading to reduced repressor levels. Therefore, two point mutations that disrupt RicR binding to DNA (11) were introduced into the *ricR* promoter (*ricRp*) of the $ricR_{C38A}$ construct to allow the constitutive expression of the $ricR_{C38A}$ allele (p^c-$ricR_{C38A}$) (see Table 1 and Materials and Methods). This allele was introduced into a CDC1551 *ricR* mutant on a plasmid that integrates into the *attB* site of the *M. tuberculosis* chromosome. As a control, we also introduced a plasmid expressing WT *ricR* from $ricRp^c$ (p^c-$ricR^+$) into the *ricR* mutant.

Expression of p^c-$ricR_{C38A}$ resulted in extreme sensitivity to Cu (Fig. 7B, far right). Interestingly and in contrast, the p^c-$ricR^+$-expressing strain was hyperresistant to Cu (Fig. 7B). Because RicR is a Cu-binding protein, it is possible that it sequesters Cu and protects against Cu toxicity when constitutively overproduced. Importantly, we found that the p^c-$ricR_{C38A}$-expressing strain, but not the p^c-$ricR^+$-expressing strain, was highly attenuated in mice (Fig. 7C). Taken together, our data support a model where several gene products of the RicR regulon, perhaps including RicR itself, are likely critical for WT Cu resistance and full virulence in a mouse model of infection.

It is worth noting that although a *ricR* null mutant is hyperresistant to Cu *in vitro*, it is not hypervirulent in mice. This mutant

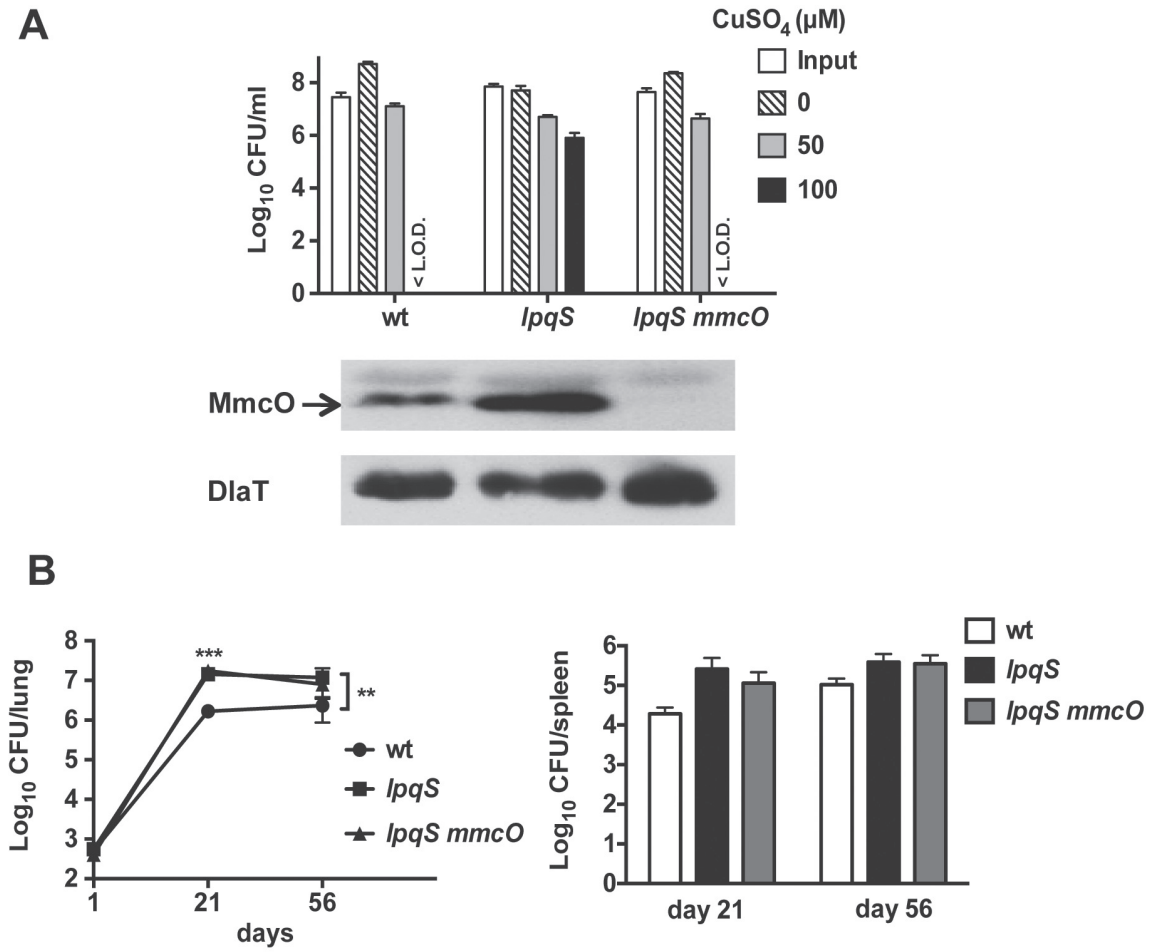

FIG 4 Overexpression of *mmcO* resulted in high Cu resistance. (A) Deletion of *mmcO* from the *lpqS* mutant resulted in WT Cu resistance. A Cu sensitivity assay (top) was performed with the WT strain and the *lpqS* and *lpqS mmcO* mutant strains. Data are representative of two experiments each done in triplicate. <L.O.D., below the limit of detection (which was 100 CFU). Immunoblot analysis (bottom) shows that disruption of *mmcO* in the *lpqS mmcO* strain eliminates MmcO protein from this strain. Data are representative of three biological replicates. (B) Deletion of *mmcO* from the *lpqS* mutant did not alleviate the hypervirulence phenotype. The CFU counts in the lungs and spleens of C57BL/6 mice infected with the WT, *lpqS*, and *lpqS mmcO* strains are plotted. The initial dose of infection was ~500 CFU/mouse. Measurements were made on days 1 ($n = 3$), 21 ($n = 4$), and 56 ($n = 4$). Two-way analysis of variance and a Bonferroni posttest showed that at day 21, both the *lpqS* and *lpqS mmcO* mutant strains were statistically significantly different from the WT strain (***, $P < 0.001$) and that at day 56, the *lpqS* mutant strain was statistically significantly different from the WT strain (**, $P < 0.01$). The data represent the mean ± standard deviation of a typical experiment that was done twice.

had a subtle growth defect in the lungs of infected mice compared to the WT and *ricR*-complemented strains (Fig. 7C, right panel).

Finally, we tested if *M. tuberculosis* strains defective in proteasome function were more sensitive to Cu. Proteasomal degradation of a protein requires PafA (proteasome accessory factor A), which ligates the posttranslational modifier Pup (prokaryotic ubiquitin-like protein) to protein substrates, and Mpa (mycobacterial proteasome ATPase), which delivers pupylated protein substrates into the proteasome core for degradation (reviewed in reference 27). Mutations that reduce proteasomal degradation have repressed the expression of all RicR regulon genes; however, RicR itself does not appear to be a proteasome substrate (11). Nonetheless, because the RicR regulon is repressed in proteasome-defective *M. tuberculosis* strains, we predicted that these strains would be more sensitive to Cu than WT bacteria are. Indeed, we found that *M. tuberculosis* strains lacking *mpa* or *pafA* were more sensitive to Cu than the WT and complemented strains were (Fig. 7D). Taken together, this suggests that the *in vivo* attenuated phenotype of proteasomal degradation mutants could be partly due to reduced Cu resistance.

DISCUSSION

The discovery of several Cu-responsive regulons in a human-exclusive pathogen suggests that *M. tuberculosis* faces host-supplied Cu during infections. In this work, we sought to understand the contribution of the RicR regulon to Cu resistance and virulence in mice. We determined that, with the exception of *mymT*, the disruption of any single RicR-regulated gene was insufficient to sensitize *M. tuberculosis* to Cu. We also established in this study that *mmcO* is a member of the RicR regulon. Overexpression of *mmcO* resulted in Cu hyperresistance but did not impact virulence. We also determined that the contributions of individual RicR-regulated genes to pathogenesis appear to be minimal. Single mutations in *mmcO*, *lpqS*, Rv2963, and *socA* did

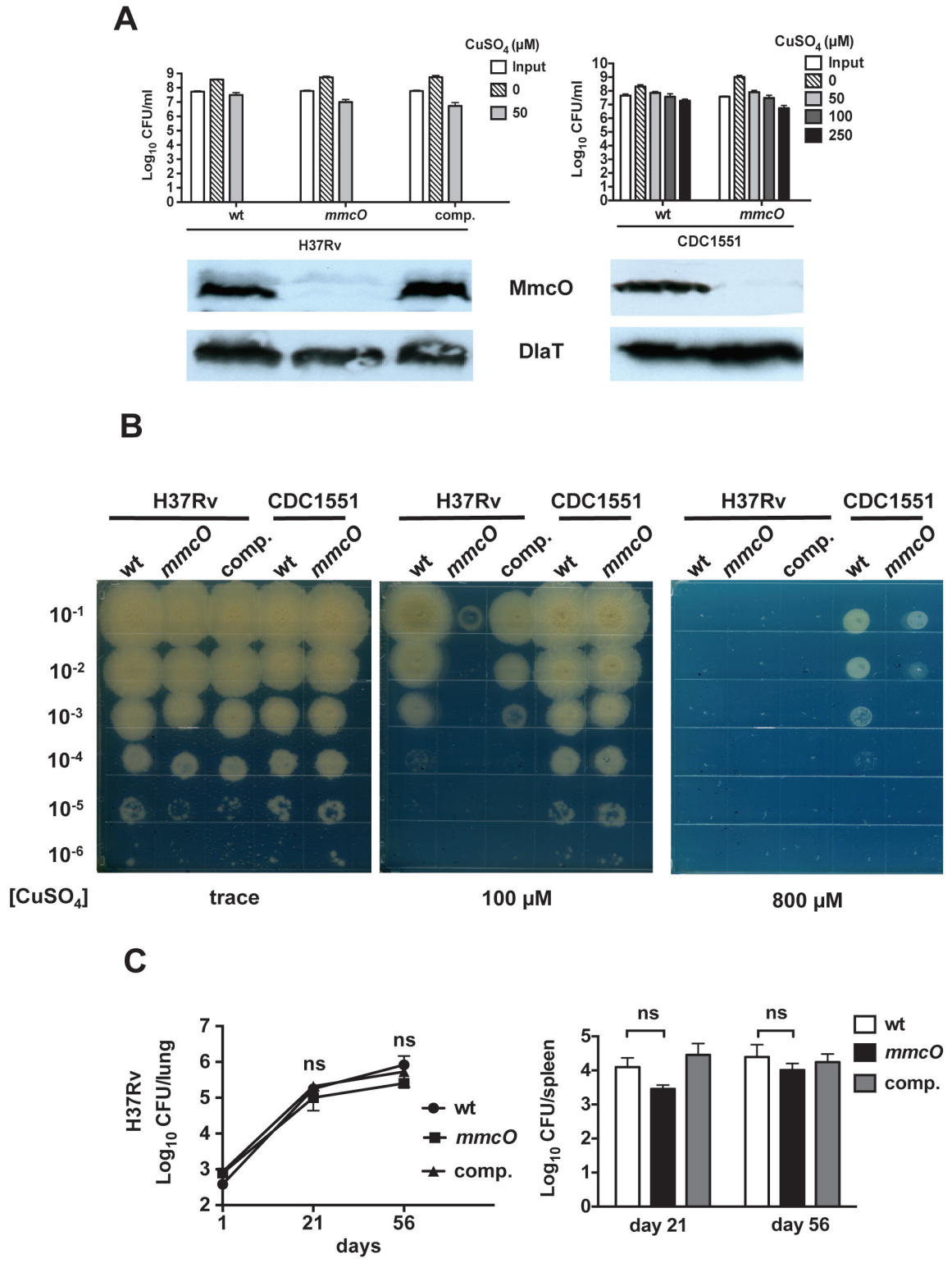

FIG 5 Deletion of *mmcO* had no effect on virulence. (A) Cu sensitivity assay (top) of WT and *mmcO* mutant strains in the H37Rv and CDC1551 backgrounds. We also complemented the *mmcO* mutation in the H37Rv background. This is representative of two independent experiments, each done in triplicate. At 150 μM, no CFU of the H37Rv strains were detected. Immunoblot analysis (bottom) of the same strains with polyclonal antibodies to MmcO. Antibodies to dihydrolipoamide acetyltransferase (DlaT) were used to show even loading of cell lysates. (B) Agar plate assay assessing the Cu sensitivity of the *M. tuberculosis* strains in panel A. Serial dilutions of *M. tuberculosis* cultures were spotted onto agar with the indicated $CuSO_4$ concentrations. Data are representative of two independent experiments. (C) CFU counts in the lungs and spleens of C57BL/6 mice infected with WT, *mmcO*, and *mmcO*-complemented (comp.) *M. tuberculosis* strain H37Rv. The results shown are for days 1 ($n = 3$), 21 ($n = 4$), and 56 ($n = 4$). ns, not significant. The data represent the mean ± SD of a typical experiment that was done twice.

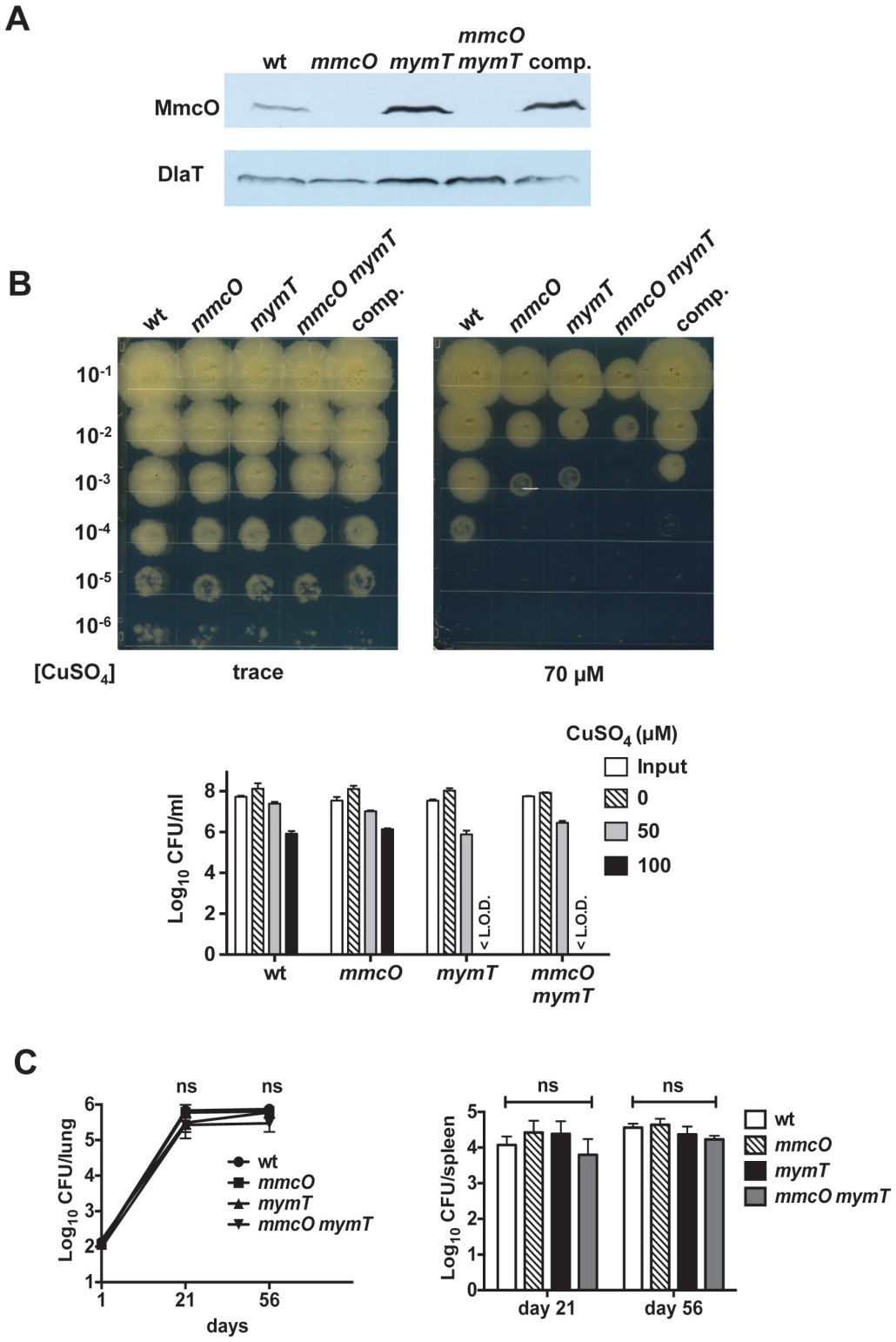

FIG 6 Deletion of both *mmcO* and *mymT* was not sufficient to attenuate *M. tuberculosis in vivo*. (A) Immunoblot analysis shows that MmcO protein was absent from an *mmcO mymT* double mutant. The WT, *mmcO*, *mymT*, *mmcO mymT*, and complemented (comp.) double mutant strains were used. Whole-cell lysates were analyzed with antibodies to MmcO. Dihydrolipoamide acetyltransferase (DlaT) was used as a loading control. (B) Agar plate assay (top) determining the Cu susceptibility of the WT, *mmcO*, *mymT*, *mmcO mymT*, and complemented double mutant strains. Data are representative of two independent experiments. The results of a liquid-based Cu sensitivity assay of the aforementioned *M. tuberculosis* strains are also shown (bottom). Data are representative of two experiments done in triplicate. (C) CFU counts in the lungs and spleens of C57BL/6 mice infected with the WT and *mmcO*; *mymT*, and *mmcO mymT* mutant *M. tuberculosis* strains. The results shown are for days 1 ($n = 3$), 21 ($n = 4$), and 56 ($n = 4$). The data represent means ± standard deviations. ns, not significant.

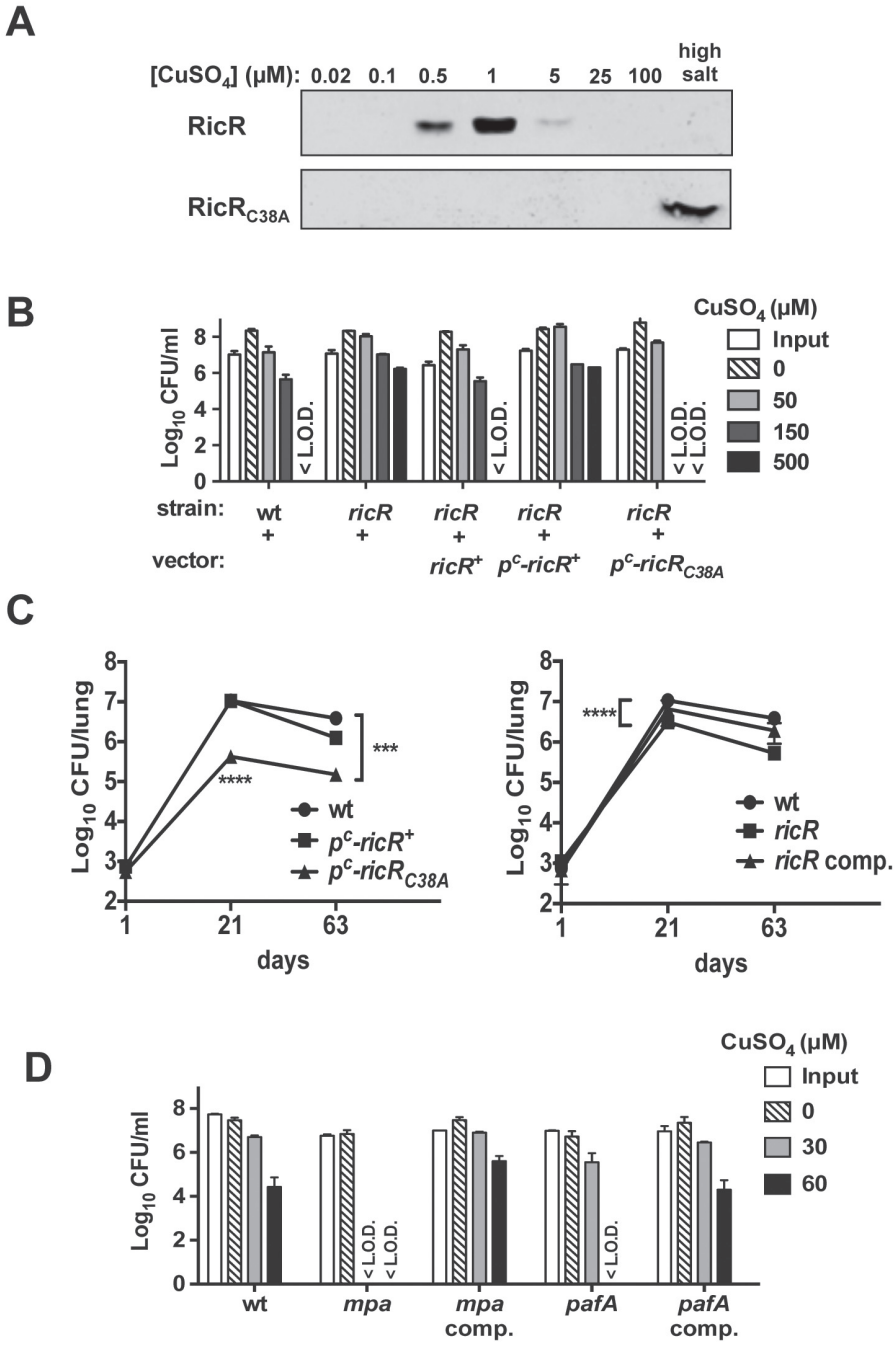

FIG 7 Repression of the entire RicR regulon sensitized *M. tuberculosis* to Cu and attenuated *M. tuberculosis* in mice. (A) DNA affinity chromatography shows that RicR dissociates from the *lpqS* promoter in the presence of Cu as previously described (top) while $RicR_{C38A}$ constitutively bound to DNA regardless of the presence of Cu (bottom). Protein was eluted from the DNA with sequential increasing amounts of $CuSO_4$ or a high salt concentration (last). (B) A Cu sensitivity assay revealed that the p^c-$ricR_{C38A}$ strain was hypersensitive to Cu. The WT CDC1551/pMV306 (empty vector), *ricR*/pMV306, *ricR*/pMV-$ricR^+$, *ricR*/pMV-p^c-$ricR^+$, and *ricR*/pMV-p^c-$ricR_{C38A}$ strains were tested for Cu sensitivity. Data represent the mean ± standard deviation of one experiment that was done three times. (C) Constitutive repression of the *ricR* regulon attenuated *M. tuberculosis* growth in mice. CFU counts in the lungs of C57BL/6 mice infected with the WT/pMV306, *ricR*/pMV-p^c-$ricR^+$, *ricR*/pMV-p^c-$ricR_{C38A}$, *ricR*/pMV306, or *ricR*/pMV-$ricR^+$ (*ricR* comp.) strain are shown. The data were separated into two graphs for clarity but represent infections done within the same week. The WT infection in both panels represents the same experiment. The initial dose of infection was ~500 CFU/mouse. The data represent the means ± standard deviations at days 1 ($n = 3$), 21 ($n = 4$), and 63 ($n = 4$) postinfection. The WT and p^c-$ricR_{C38A}$ data are representative of two independent infections. ****, $P < 0.0001$; ***, $P < 0.001$ (two-way analysis of variance with a Bonferroni posttest). (D) Proteasomal-degradation-defective strains were hypersensitive to Cu. The WT/pMV306 and *mpa*/pMV306, *mpa*/pMV-mpa^+, *pafA*/pMV306, and *pafA*/pMV-$pafA^+$ mutant H37Rv strains were exposed to Cu for 10 days. The data represent the means ± standard deviations of one typical experiment that was done three times. <L.O.D., below the limit of detection.

not attenuate *M. tuberculosis* growth in mice, and curiously, *lpqS* and Rv2963 mutants were hypervirulent for reasons that remain to be determined. We also tested the idea that deletion of the two RicR-regulated genes directly implicated in Cu resistance (*mymT*, *mmcO*) might have a more robust effect on bacterial survival *in vivo*. However, an *mmcO mymT* mutant was as virulent as WT *M. tuberculosis* in mice.

We did not detect robust Cu-associated phenotypes with the *lpqS* and Rv2963 mutants. LpqS and Rv2963 are putative membrane proteins each predicted to have several histidines that localize just outside the cytoplasmic membrane. These residues may be potential candidates to coordinate metal ions. Rv2963 is predicted to be a permease the disruption of which may perhaps alter either the import or the export of Cu or other metal ions under certain conditions.

It has been reported that a Δ*lpqS*::*hyg* mutant is hypersensitive to Cu *in vitro* (28). As in our study, the authors of that previous study could not complement their mutation, suggesting that their Cu-sensitive phenotype might be unlinked to LpqS. A possibility is that the disruption of *lpqS* in the study of Sakthi and Narayanan was polar on the expression of genes that are important for Cu resistance. Two uncharacterized genes, *cysK2* and Rv0849, are cotranscribed with *lpqS* and may perhaps have a role in Cu resistance. Another possibility is that disruption of *lpqS* results in the dysregulation of the divergently expressed gene *mmcO*, which is important for Cu resistance. The mechanism of RicR repression of *mmcO* expression is not fully understood but may involve the bending of DNA between *mmcO* and *lpqS* to simultaneously repress both genes with a single RicR-binding site. On the basis of the published *lpqS* data and our data, we strongly hypothesize that LpqS itself is not critical for Cu resistance.

socAB is perhaps the most mysterious RicR-regulated locus; these genes are found only in the *M. tuberculosis* complex and do not resemble sequences in any other organism sequenced to date. Because of the lack of robust phenotypes associated with disruptions in this locus, it is unclear what role it plays, if any, in Cu homeostasis.

Although the *lpqS* and Rv2963 transposon mutants lacked clear Cu resistance phenotypes, both had very intriguing hypervirulence phenotypes in mice; however, we could not complement these mutations to restore WT virulence. We hypothesized that overexpression of *mmcO* was responsible for the hypervirulence of the *lpqS* strain, but this was not the case (Fig. 4). Currently, we can only speculate as to why the *lpqS* and Rv2963 mutants are hypervirulent. A possibility is that the absence of these putative membrane proteins permits the bacteria to grow more rapidly *in vivo*, which appears to be the reason for the increased virulence of these strains. Alternatively, it is possible that mutant or truncated proteins that alter the course of infection are produced by these strains. Yet another possibility is that the transposon insertions in these mutants change the expression of other genes that increase the growth of the bacteria *in vivo*. Needless to say, we are very interested in understanding why these mutants rapidly kill their hosts and are testing several of these hypotheses.

None of the genes of the RicR regulon individually showed a role in promoting virulence. It is possible that in the absence of one or more of the RicR-regulated genes, other genes encoding Cu-binding proteins or efflux systems could be induced to compensate for their absence. Nonetheless, a *ricR* mutant that constitutively represses all RicR-dependent promoters was highly attenuated in mice. These data strongly suggest that the RicR regulon, either in its entirety or in part, is required for the full virulence of *M. tuberculosis*. Our *in vitro* and *in vivo* data also suggest that RicR itself may sequester Cu like a metallothionein because the constitutive overexpression of WT *ricR* (as opposed to $ricR_{C38A}$, which is expected not to bind Cu) resulted in increased Cu resistance (Fig. 7B). Another possible reason that single mutations had little to no impact *in vivo* is that mice may not be the best model for testing the role of these genes; some genes may show importance in models of infection that more closely resemble human disease.

It is notable that we observed considerable differences in Cu susceptibility, depending on the assay we used. We observed robust differences in Cu resistance when using the liquid-based quantitative assay for the *mymT* (hypersensitive), *ricR*, and *lpqS* (hyperresistant) mutant strains, while in contrast, we could detect a phenotype for *mmcO* mutants only by using an agar plate-based assay. Interestingly, both assays revealed that *M. tuberculosis* strain CDC1551 is inherently more resistant to Cu than H37Rv is. The results from the different assays suggest that different Cu-binding proteins are important under different conditions. Furthermore, it has yet to be determined which Cu regulon, CsoR or RicR, responds first to Cu stress. It is also possible that the repressors respond to different concentrations of Cu. In the agar-based assay, bacteria were exposed to Cu throughout the experiment (14 to 21 days), whereas in the liquid-based assay we exposed the bacteria for a defined time period (10 days) before inoculation onto agar. Additionally, the oxygen tension, which has a critical impact on the redox status of Cu, could impact the effective Cu^+ concentration during the experiment. Finally, the media used for agar versus broth cultures are also slightly different and may impact Cu susceptibility in unknown ways.

At the forefront of our remaining questions is what the link is between RicR regulon expression and *M. tuberculosis* proteasomal degradation. A simple explanation would be that RicR is a proteasome substrate and that the accumulation of this repressor in proteasome degradation-defective mutants results in repression of the regulon. However, we have no evidence that RicR accumulates in proteasome-defective mutants. Another possibility is that one or several Cu-binding proteins are proteasome substrates the accumulation of which in a proteasome degradation-defective strain sequesters Cu away from RicR, leading to gene repression. These and other hypotheses are currently being tested.

Our work supports previous observations that Cu homeostasis is critical for the pathogenesis of *M. tuberculosis* (5, 10). As in other organisms, too little accessible Cu is detrimental while too much Cu can be toxic. Taken together, our findings affirm that the careful control of Cu homeostasis is essential for *M. tuberculosis* virulence and that the RicR regulon plays an important and nonredundant role in this process.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids, and primers. The bacterial strains, plasmids, and primers used in this work are listed in Table 1. *M. tuberculosis* strains were grown in 7H9 broth (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, 0.5% bovine serum albumin, 0.2% dextrose, and 0.085% sodium chloride (ADN) or Sauton's minimal medium (3.7 mM potassium phosphate, 2.5 mM magnesium sulfate, 30 mM L-asparagine, 3.5 mM zinc sulfate, 9.5 mM citric acid, 6.0% glycerol, 0.005% ferric ammonium citrate, 0.05% Tween 80). Cultures were grown at 37°C without agitation in vented flasks. For *M. tuberculosis* growth on solid medium, Middlebrook 7H11 agar (Difco) was prepared with

Middlebrook OADC (oleic acid, albumin, dextrose, and catalase; BBL) supplementation. *M. tuberculosis* strains were grown in 50 μg ml^{-1} kanamycin, 50 μg ml^{-1} hygromycin, and/or 25 μg ml^{-1} streptomycin when necessary. *Escherichia coli* cultures were grown in Luria-Bertani (LB) broth (Difco) or on LB agar at 37°C. Antibiotics were added at final concentrations of 100 μg ml^{-1} kanamycin, 150 μg ml^{-1} hygromycin, and 50 μg ml^{-1} streptomycin.

To clone the p^c-$ricR_{C38A}$ allele into pMV306, site-directed mutagenesis was performed by splicing by overlap extension by PCR (29) (the primers used are described in Table 1). Cysteine 38 of RicR was first changed to alanine. A second mutation was then introduced into the $ricR_{C38A}$ plasmid; 2 nucleotides (nt) were changed in the *ricR* promoter (*ricRp*) (TAC CCCGCTGGGTA → TACCCCGCTGTTTA = $ricRp^c$) to reduce repressor binding.

With the exception of the biotin tetraethylene glycol (BioTEG; Operon)-labeled primer, all primers were purchased from Invitrogen (Table 1). Phusion polymerase and restriction enzymes from New England Biolabs were used for cloning. All plasmid inserts generated by PCR were sequenced by GENEWIZ (Plainfield, NJ).

Production of MmcO antibodies and MmcO immunoblot analysis. For antibody production, MmcO-His_6 was overproduced in *E. coli* and purified under denaturing conditions by following the manufacturer's instructions (Qiagen). Polyclonal rabbit antibodies were generated by Covance (Denver, PA). DlaT (used for loading controls) and RicR antibodies were described previously (11, 30, 31).

For immunoblot analysis, equivalent cell numbers based on optical density at 580 nm (OD_{580}) were collected for each relevant strain at the same growth phase. For Cu-treated protein samples, bacteria were treated with $CuSO_4$ for 4 h at a final concentration of 500 μM. Bacteria were washed once with phosphate-buffered saline (PBS) with 0.05% Tween 80 and resuspended in 300 μl of lysis buffer (100 mM Tris-Cl, 1 mM EDTA, pH 8). Bacteria were lysed by bead beating with zirconia beads three times for 30 s each. Whole-cell lysates were mixed with 4× sodium dodecyl sulfate (SDS) sample buffer and boiled for 10 min at 100°C. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated with rabbit polyclonal antibodies to MmcO at a dilution of 1:1,000 in 3% BSA in Tris-buffered saline with Tween 20, and visualized with horseradish peroxidase coupled with anti-rabbit secondary antibodies (Thermo Scientific). Detection of horseradish peroxidase was performed with SuperSignal West Pico or West Femto Chemiluminescent Substrate (Thermo Scientific).

Construction of *M. tuberculosis* mutants. We made *M. tuberculosis* mutants by using a method previously described by our lab (11). Briefly, pYUB854, which was originally developed for specialized transduction mutagenesis (32), was used to clone ~700 bp of sequence both upstream (5′) and downstream (3′) of a gene of interest. The 5′ and 3′ sequences, including the start and stop codons, respectively, were cloned to flank the hygromycin resistance cassette in pYUB854. We also constructed a kanamycin resistance-marked version of this plasmid to make the Δ*mmcO*::*kan* mutant. The hygromycin resistance cassette in pYUB854 was replaced with the kanamycin resistance cassette from pMV306.kan to generate pYUB854.kan (the primers and plasmids used are described in Table 1). Because the *ricR* mutant is in the CDC1551 background, we chose to mutate *mmcO* in both the H37Rv and CDC1551 strains.

Plasmids were digested with PacI, and ~1 μg of linearized, gel-purified DNA was used for electroporation into *M. tuberculosis*. *M. tuberculosis* strains were grown to an OD_{580} of ~0.4 to 1, washed, and resuspended in 10% glycerol to make electrocompetent cells as described in detail elsewhere (33). Bacteria were inoculated onto 7H11 solid medium with 50 μg ml^{-1} hygromycin or 50 μg ml^{-1} kanamycin as needed. A no-DNA control electroporation was always done to check for spontaneous antibiotic-resistant mutants. After 2 weeks, colonies were picked and inoculated into 200 μl of 7H9 medium with antibiotics. The 200-μl 7H9 starter cultures were inoculated into 5-ml cultures for further analysis. Potential mutants were tested by immunoblot analysis, PCR (Taq polymerase; Qiagen), and sequencing.

DNA affinity chromatography. Experiments were performed as described previously (11). DNA probes were made by amplifying the *lpqS* promoter with a forward primer containing 5′ BioTEG (Operon) modifications. Clarified lysates of *E. coli* producing either WT RicR or $RicR_{C38A}$ were incubated with a DNA-Dynabead mixture. Protein interacting with the coupled DNA was sequentially eluted with increasing concentrations of $CuSO_4$ as indicated and finally with high-salt buffer (1 M NaCl in 50 mM Tris, pH 7.5). RicR immunoblotting was performed as previously described (11).

Genome sequencing. MHD131 (*lpqS*::ΦMycoMarT7) and the parental H37Rv strain were sequenced with an Illumina GenomeAnalyzer IIx. Approximately 5 μg of DNA was processed by the standard Illumina sample preparation protocol for genomic DNA (Illumina, Inc.), and the samples were sequenced in paired-end mode with a read length of 51 nt. The genomes were assembled by mapping reads to the public H37Rv reference sequence (GenBank accession no. NC_000962) and single-nucleotide polymorphisms and insertions/deletions as described in reference 34. The mean depths of coverage (number of reads covering each site) were 59.6 and 63.1 times, respectively, for the two strains.

Cu sensitivity assays. $CuSO_4$ powder was dissolved in water and sterilized through 0.45-μm-pore-size filters to make a 1 M stock solution. *M. tuberculosis* strains were grown in 7H9-ADN medium to an OD_{580} of ~0.5 to 1. Bacteria were harvested, washed once with Sauton's medium (no added Cu), and then subjected to a low-speed spin (800 *g* for 8 min) to remove clumped cells. Bacteria were diluted to an OD_{580} of 0.08 in Sauton's medium. Each culture was placed into the wells of a 96-well plate at 194 μl per well and treated with 6 μl of $CuSO_4$ at different concentrations, and the plate was incubated for 10 days. Bacteria were then diluted and inoculated onto 7H11 agar and CFU were enumerated 2 to 3 weeks later. Because we noticed some variability in the minimum bactericidal Cu concentration between experiments, we always used a range of Cu concentrations for every experiment and drew conclusions based on reproducible trends. All experiments were done at least in duplicate.

The agar plate assay was performed as described previously (15, 17). *M. tuberculosis* strains were grown in 7H9-ADN medium to an OD_{580} of ~0.5 to 1. Cells were harvested by centrifugation, washed once with PBS-Tween (0.05%), and then spun slowly at 800 × *g*. Declumped cell suspensions were diluted to an OD_{580} of 0.1 with PBS-Tween. Three-microliter samples of serial dilutions were spotted onto *M. tuberculosis* 7H11-OADC agar plates containing either no additional $CuSO_4$ or different concentrations thereof. Plates were incubated at 37°C for ~2 weeks.

Mouse infections. Mouse infections were performed essentially as described previously (12). *M. tuberculosis* strains were grown in 7H9-ADN medium to an OD_{580} of ~0.4. Bacterial clumps were removed from the cultures as described above. Female C57BL6/J mice (Jackson Laboratories) were infected with a Glas-Col Inhalation Exposure System to inoculate ~200 to 400 CFU/mouse. Three (day 1) or four (days 21 and ~56) mice were humanely sacrificed, and their lungs and spleens were removed, homogenized, and inoculated onto 7H11 agar. CFU were enumerated after 2 to 3 weeks. All procedures were performed with the approval of the NYU Institutional Animal Care and Use Committee.

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