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; a Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas, USA;

* 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.
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.

mymT overexpression results in Cu hyperresistance but no hypervirulence. Upon closer inspection of the lpqS locus, we hypothesized that the transposon insertion in the lpqS mutant somehow increased the expression of the diversely expressed gene mymT (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 Cu²⁺ or Fe²⁺, as well as phenolic compounds (23–26). Recently it was shown that MmcO can oxidize Fe²⁺ to Fe³⁺ and perhaps can also convert Cu²⁺ to Cu³⁺ (17). Additionally, Rowland and Niederweis observed the induction of MmcO production in M. tuberculosis upon Cu treatment (17). A microarray analysis determined that mmpC 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 mmpC 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 mmpC 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 mmpC 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 mmpC, 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 mmpC 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 mmpC from WT M. tuberculosis. We deleted and disrupted mmpC 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 CuSO₄ for the CDC1551 strains in our assays. Nonetheless, in both strain backgrounds, the mmpC mutants showed WT Cu resistance in our quantitative Cu susceptibility assay (Fig. 5A). However, during the preparation of this report, Rowland and Niederweis showed that an mmpC 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 mmpC mutants were more sensitive to Cu than the WT or complemented strain was (Fig. 5B).

To determine the importance of mmpC in vivo, we infected mice with the H37Rv WT, mmpC mutant, and mmpC-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\(^{2+}\) 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.
| Strain, plasmid, or primer | Phenotype, genotype, or sequence | Source or reference |
|---------------------------|---------------------------------|--------------------|
| **M. tuberculosis strains** |                                 |                    |
| H37Rv                    | WT                              | ATCC 25618          |
| MHD118                   | Hyg' WT/pMV306                  | 12                 |
| MHD22                    | Kan' Hyg' ΔmmpC::ΔMycoMarT7/pMV306 | 12                |
| MHD23                    | Kan' Hyg' ΔmmpC::ΔMycoMarT7/pMV-mpa | 12              |
| MHD62                    | Kan' Hyg' pafA::ΔMycoMarT7/pMV306 | 16                |
| MHD63                    | Kan' Hyg' pafA::ΔMycoMarT7/pMV-pafA | 16              |
| MHD113                   | Kan' ΔlpqS::ΔMycoMarT7 (transposon inserted in codon 44) | This work         |
| MHD118                   | Kan' ΔlpqS::ΔMycoMarT7 (transposon inserted in codon 351) | This work         |
| MHD696                   | Kan' ΔsoA::ΔMycoMarT7 (transposon inserted in codon 22) | This work         |
| MHD701                   | Hyg' ΔmynT::hyg                 | 15                 |
| MHD702                   | Hyg' ΔmynT::hyg/pMV306.kan-mymT  | 15                 |
| MHD752                   | Hyg' ΔmymC::hyg                 | This work          |
| MHD764                   | Kan' Hyg' ΔmmpC::ΔMycoMarT7 ΔmmyC::Δhyg | This work | |
| MHD794                   | Kan' ΔwpC/pMV306.kan             | This work          |
| MHD795                   | Kan' ΔmmyC::hyg/pMV306.kan       | This work          |
| MHD796                   | Kan' ΔmmyC::hyg/pMV306.kan-mmcO  | This work          |
| MHD840                   | Kan' ΔmynT::hyg ΔmmyC::kan       | This work          |
| MHD867                   | Kan' ΔmynT::hyg ΔmmyC::kan/pMVstrep-mmcO mmyT | This work         |
| CDC1551                  | WT                              | W. Bushai lab collection |
| MHD557                   | Kan' ricR::ΔMycoMarT7           | 14                 |
| MHD583                   | Hyg' WT/pMV306                  | 11                 |
| MHD589                   | Kan' Hyg' ricR::ΔMycoMarT7/pMV306 | 11              |
| MHD590                   | Kan' Hyg' ricR::ΔMycoMarT7/pMV-ricR | 11            |
| MHD694                   | Kan' Hyg' ricR::ΔMycoMarT7/pMV-ricR ΔC38A | This work      |
| MHD707                   | Kan' Hyg' ricR::ΔMycoMarT7/pMV-ricR Δ-ricR ΔC38A | This work  |
| MHD708                   | Kan' Hyg' ricR::ΔMycoMarT7/pMV-ricR Δ-ricR ΔC38A | This work  |
| MHD755                   | Hyg' ΔmymC::hyg                 | This work          |
| **E. coli strains**       |                                 |                    |
| DH5α                      | F^- 80d lacZ ΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (rK- mK+) phoA supE44 λ-thi- GIBCO BRL | 1 gyro96 relA |
| ER2566                    | F^- λ F' fsuA2 [lon] ompT lacZ:77 gene1 gal salA11 Δ(mcrC-mrr)114::ΔS10 R(mcr-c73::miniTn10)2 | 35 |
|                           | (Tet') endA1 [dcm]              |                    |
| **Plasmids**              |                                 |                    |
| pET24b (+)                | Kan', for production of C-terminally His6 epitope-tagged protein | Novagen  |
| pET24b (+)-mmyC          | Kan', for production of MmyC-His6 in E. coli | This work    |
| pET24b (+)-ricR          | Kan', for production of untagged RicR in E. coli | This work    |
| pET24b (+)-ricR ΔC38A    | Kan', for production of untagged RicR ΔC38A in E. coli | This work    |
| pMV306.kan              | Kan', integrates at attB site in mycobacterial chromosome | 36        |
| pMV306                 | Hyg', integrates at attB site in mycobacterial chromosome | 36        |
| pMV306.strep            | Strep', integrates at attB site in mycobacterial chromosome | Gift from J. McKinney lab |
| pMV306.kan-mmcO         | Kan', for complementation of mmyC mutant | This work |
| pMV-ricR                | Hyg', for complementation of ricR mutant | 11        |
| pMV-ricR ΔC38A          | Hyg', WT ricR expressed from mutated ricR (see text) | This work |
| pMV-ricR ΔC38A          | Hyg', ricR ΔC38A allele expressed from mutated ricR | This work |
| pMVstrep-mmcO mmyT      | Strep', for complementation of mmyC mmyT double mutant | This work |
| pYUB854                 | Hyg', allelic-exchange vector | 32        |
| pYUB854.kan             | Kan', allelic-exchange vector | This work |
| pYUB854-mmcO           | Hyg', M. tuberculosis ΔmmyC::hyg (deletion-disruption plasmid) | This work |
| pYUB854.kan-mmcO       | Kan', M. tuberculosis ΔmmyC::kan (deletion-disruption plasmid) | This work |

**Primers**

- **Ndel** Rv0846c F1: GGGCATATGCCGCGATGGGCCAGCGGGGTAAAC
- **XhoI** Rv0846c R1: GGCTCGAGCACGAAATGGTACTCCGACGGGTTGC
- **Rv0846cF2seq**: GCCAGCGGAGCCCGCGACTCGAGACCATC
- **Rv0846cR2seq**: CCGCGTTCAGCGGATGGCGAACCGG
- **Kpn1 846cMutant F1**: GAGGGTACCTATCTGCGGTTGGAGGTATGCTGGT
- **XbaI 846cMutant R1**: GTTACGCGCTGGTGTAGCAGGATGCGGTC
- **HindIII 846cMutant F2**: ACCCGCGTGGCGAAGGCTTTTACACTTGAGTCAAGCGG
- **SpeI 846cMutant R2**: TCAGAGGCTTCGATGGTACTAGTGAGATGCCGTAACC

(Continued on following page)
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 than the single mutants.
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/H11001 (9). Cysteine 38 of RicR is predicted to be required for Cu/H11001 binding (D. Giedroc, personal communication); thus, conversion of cysteine 38 to alanine (RicR C38A) would prevent RicR from sensing Cu/H11001 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 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 ricR C38A (pc - ricR C38A) into the ricR mutant. Expression of pc - ricR C38A resulted in extreme sensitivity to Cu (Fig. 7B, far right). Interestingly and in contrast, the pc - ricR C38A-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 ricR C38A-expressing strain, but not the p ricR C38A-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...
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...
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 CuSO₄ 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.
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. (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<sub>C38A</sub> constitutively bound to DNA regardless of the presence of Cu (bottom). Protein was eluted from the DNA with sequential increasing amounts of CuSO₄ or a high salt concentration (last). (B) A Cu sensitivity assay revealed that the p<sup>p</sup>*-ricR<sub>C38A</sub> strain was hypersensitive to Cu. The WT CDC1551/pMV306 (empty vector), ricR/pMV306, ricR/pMV-ricR <sup>*</sup>, ricR/pMV-p<sup>p</sup>*-ricR <sup>*</sup>, and ricR/pMV-p<sup>p</sup>*-ricR<sub>C38A</sub> 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<sup>p</sup>*-ricR <sup>*</sup>, ricR/pMV-p<sup>p</sup>*-ricR<sub>C38A</sub>, ricR/pMV306, or ricR/pMV-ricR <sup>*</sup> (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 (<i>n</i> = 3), 21 (<i>n</i> = 4), and 63 (<i>n</i> = 4) postinfection. The WT and p<sup>p</sup>*-ricR<sub>C38A</sub> data are representative of two independent infections. ****, <i>P</i> < 0.0001; ***, <i>P</i> < 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 <sup>*</sup>, pafA/pMV306, and pafA/pMV-pafA <sup>*</sup> 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::hgy* 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 Sakhri and Narayanan was polar on the expression of genes that are important for Cu resistance. Two uncharacterized genes, *cytK2* 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 binding 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 hyper-virulence 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<sup>ΔG88A</sup>, 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 *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<sup>2+</sup> 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-ricR<sub>CRBA</sub> allele into pMY306, 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<sub>CRBA</sub> plasmid; 2 nucleotides (nt) were changed in the ricR promoter (ricR<sub>P</sub>) (TAC CCCGGTGGGTA → TACCCCCGCTGTATTA = ricR<sub>P</sub>) 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, was overproduced in *E. coli* and purified under denaturing conditions by following the manufacturer’s instructions (Qiagen). Polyclonal rabbit antibodies were generated by Co-vance (Denver, PA). DiaT (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<sub>580</sub>) were collected for each relevant strain at the same growth phase. For Cu-treated protein samples, bacteria were treated with CuSO₄ 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 4X 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 and downstream of a gene of interest. The 5′ = = 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 ΔmmcO: 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 strain, we chose to mutate *mmcO* in both the H37Rv and CDC1551 strains.

Plasmids were digested with PstI, and ~1 μg of linearized, gel-purified DNA was used for electroporation into *M. tuberculosis*. *M. tuberculosis* strains were grown to an OD<sub>580</sub> 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<sub>580</sub> of 0.1 with PBS-Tween. Three-micro liter samples of serial dilutions were spotted onto *M. tuberculosis* 7H11-OADC agar plates containing either no additional CuSO₄ 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 7H-9-ADN medium to an OD<sub>580</sub> 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<sub>580</sub> of 0.1 with PBS-Tween. Three-micro liter samples of serial dilutions were spotted onto *M. tuberculosis* 7H11-OADC agar plates containing either no additional CuSO₄ or different concentrations thereof. Plates were incubated at 37°C for ~2 weeks. All procedures were performed with the approval of the NYU Institutional Animal Care and Use Committee.

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