SUPPLEMENTARY INFORMATION

Copper resistance is essential for virulence of Mycobacterium tuberculosis

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SUPPLEMENTARY FIGURES

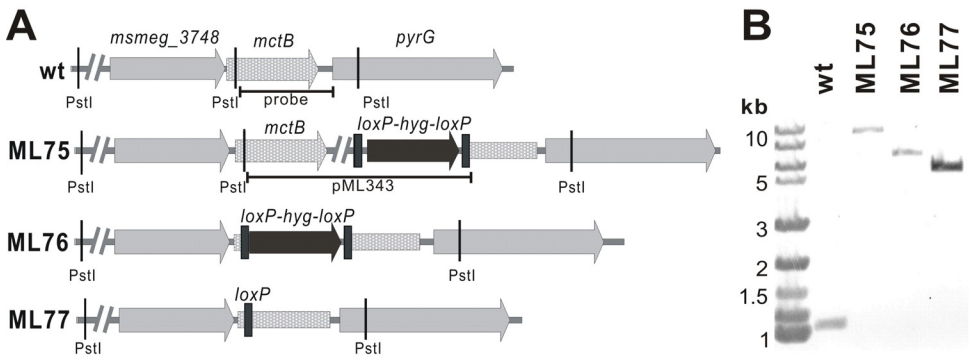

Figure S1. Construction of an *mctB* mutant of *M. smegmatis*.

A. Schematic representation of the chromosomal *ms3747* (*mctB*) region of *M. smegmatis* SMR5 (WT). A fragment of 29 bp was replaced by the 45 bp *loxP* construct in the 5' part of the gene introducing stop codons in all three reading frames to inactivate the *ms3747* gene in *M. smegmatis*. Genes are drawn to scale. The probe used for Southern blot analysis is indicated. The single crossover clone ML75 was obtained after integration of the suicide vector pML343 into the downstream region of the *ms3747* locus. Counterselection yielded the double crossover clone ML76. The *loxP*-flanked hygromycin cassette of ML76 was removed by Cre recombinase to create the unmarked mutant ML77.

B. Chromosomal DNA of *M. smegmatis* strains was digested with PstI and analyzed by Southern blotting using the probe as indicated in fig. 1A.

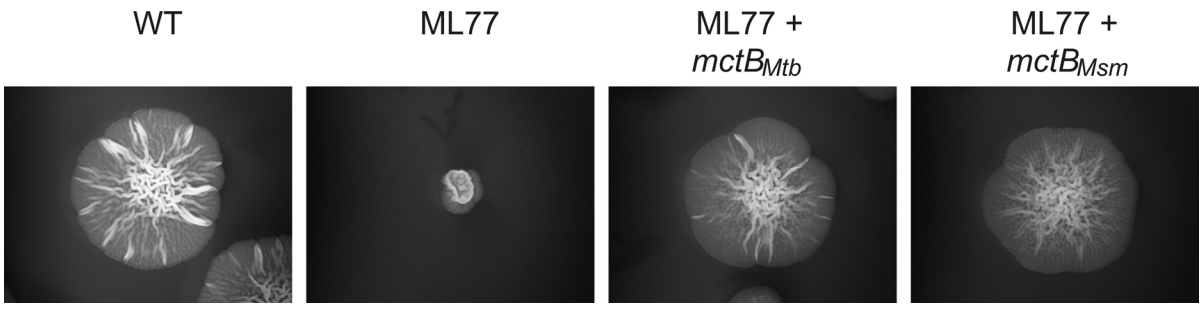

Fig. S2: Complementation of ML77 by *mctB* gene from *M. smegmatis* and *M. tuberculosis*. *MctB*$_{Mtb}$ and *mctB*$_{Msm}$ were amplified from chromosomal DNA and cloned into the mycobacterial expression vector pMS2. The resulting vectors, pMN035 and pML451 were transformed into ML77. Colonies were picked and grown over night at 37°C in 7H9 Middlebrook medium containing hygromycin. The culture was pushed through a 5 μm filter to obtain single cells and diluted to an OD_{600} of 10^{-7} of which 100 μL were plated on hygromycin-containing 7H10 plates. Colonies from single cells were photographed after 5 days at 37°C using a digital camera (AxioCam, Zeiss) attached to a stereo microscope (Stemi 2000-C, Zeiss).

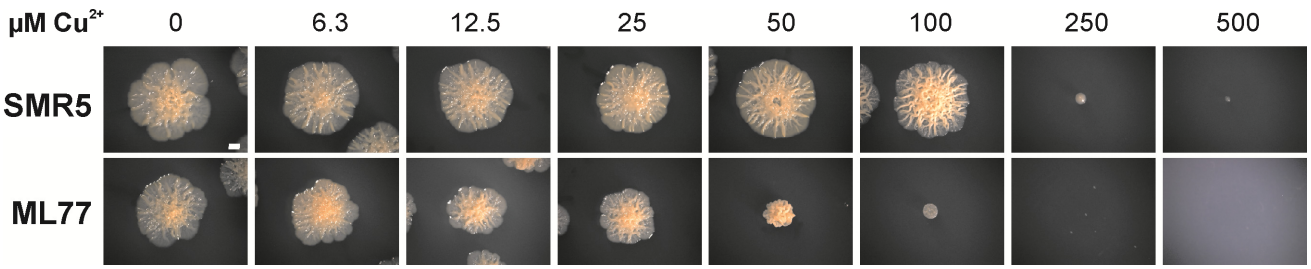

Fig. S3. Susceptibility of the *M. smegmatis ms3747* mutant to copper.

M. smegmatis WT (SMR5) and the *ms3747* mutant ML77 were grown overnight in self-made Middlebrook 7H9 medium without added copper. The culture was filtered through a 5 µm filter to obtain single cells. Appropriate dilutions were plated on self-made 7H10 agar plates supplemented with 0, 6.3, 12.5, 25, 50, 100, 250, 500 µM copper sulfate. Plates were incubated for nine days at 37°C. Pictures of representative colonies were taken using a stereomicroscope (Stemi 2000-C, Zeiss). Longer incubation times showed that *M. smegmatis* is not killed at elevated copper concentrations. Scale bar: 1 mm.

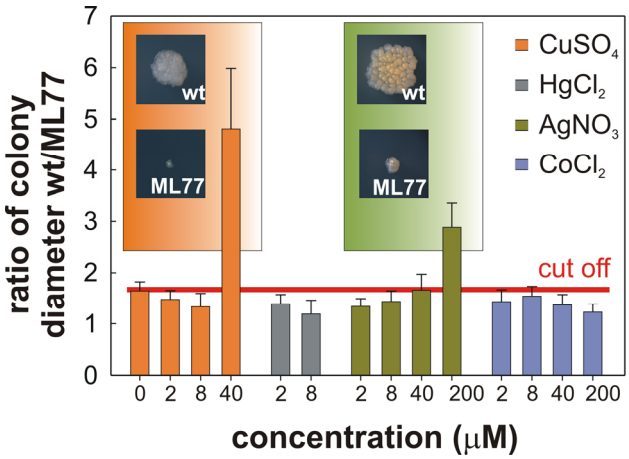

Fig. S4: Susceptibility of *M. smegmatis* Δ*mctB* mutant (ML77) to heavy metal salts.

Salts containing heavy metal ions were added to Mueller-Hinton medium at the indicated concentrations. Mid-log cultures of *M. smegmatis* were filtered through a 5 μm filter to obtain single cells, diluted to an OD_{600} of 10^{-7} and 100 µL were spread on Mueller-Hinton medium plates containing the indicated concentrations of $CuSO_4$, $HgCl_2$, $AgNO_3$ or $CoCl_2$. After five days of incubation at 37°C photographs of colonies were taken using a stereo microscope (Stemi 2000-C/AxioCam, Zeiss) at 32-fold magnification. Diameters of at least six representative colonies were measured and averaged. The average diameter of WT colonies was divided by that of the Δ*mctB* mutant (ML77). The ratios for each metal ion concentration are presented. Pictures of representative colonies are shown for the highest Cu(II) and Ag(I) concentration at which growth of the Δ*mctB* mutant ML77 was impaired.

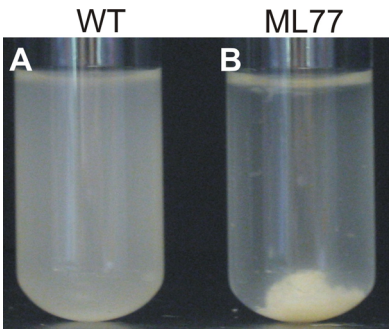

Fig. S5: Cells of the *ms3747* mutant of *M. smegmatis* aggregate in clumps.

M. smegmatis WT (**A**) and ML77 (Δ*ms3747*) (**B**) were grown on a roller drum overnight at 37 °C in Middlebrook 7H9 medium containing 0.05% Tween 80. Pictures of the test tubes were taken with a digital camera.

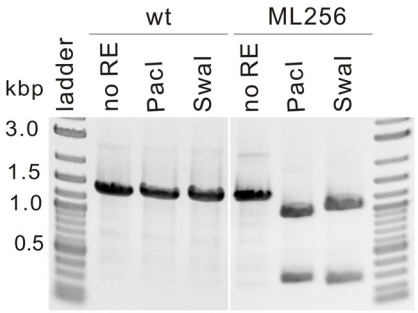

Fig. S6: Construction of the *mctB* mutant of *M. tuberculosis*.

Chromosomal DNA was isolated from *M. tuberculosis* strains and analysed by PCR using the primers CN991 and CN842 as indicated in fig. S1 A. The fragments have identical length but only the mutant fragment carries the restrictions sites SwaI and PacI which originate from the deletion vector. The genome of *M. tuberculosis* H37Rv (WT) does not contain PacI sites and only one SwaI.

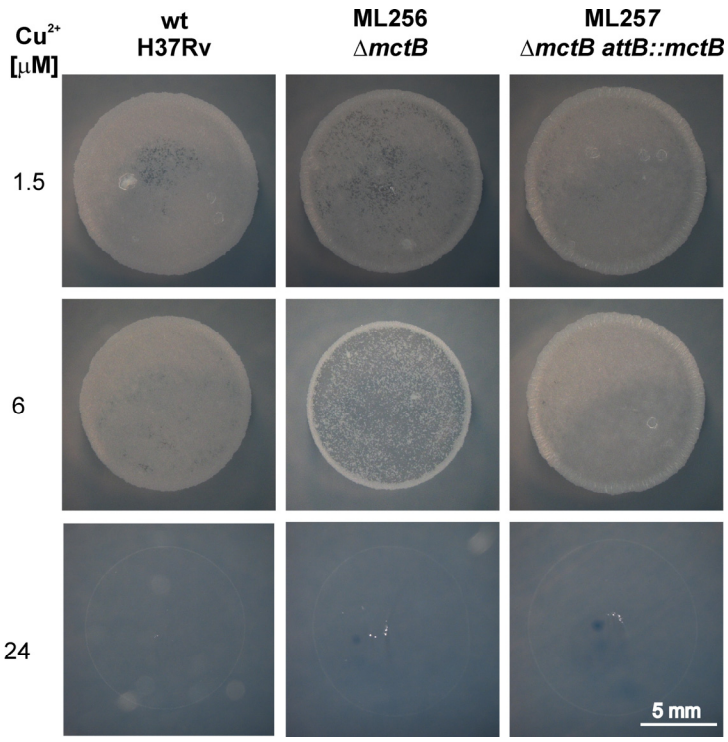

Fig. S7: Copper susceptibility of the *mctB* mutant of *M. tuberculosis*.

Pictures were taken of drops of diluted cultures of *Mtb*, ML256 (Δ*mctB*) and the $mctB_{Mtb}$ complemented strain ML257 on HdB medium plates with different $CuSO_4$ concentrations after incubation at 37°C for 22 days (magnification: 10x).

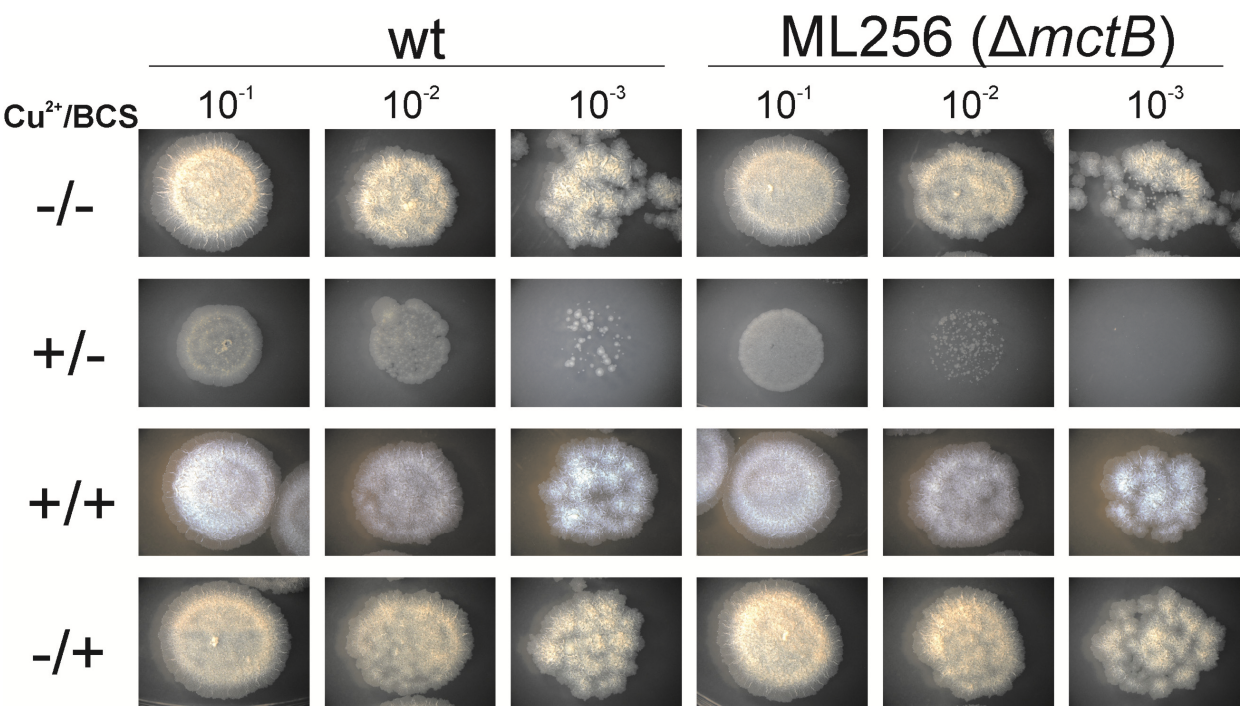

Fig. S8. MctB is required for copper resistance of *M. tuberculosis.*

Pictures were taken of drops of three dilutions of cultures of *Mtb* H37Rv, and of ML256 (Δ*mctB*) on 7H11/OADC plates. $CuSO_4$ and the Cu(I)-chelating bathocuproine disulfonate (BCS) were added at concentrations of 150 μM and 1 mM, respectively. Plates were incubated at 37°C for 28 days. Magnification: 10x.

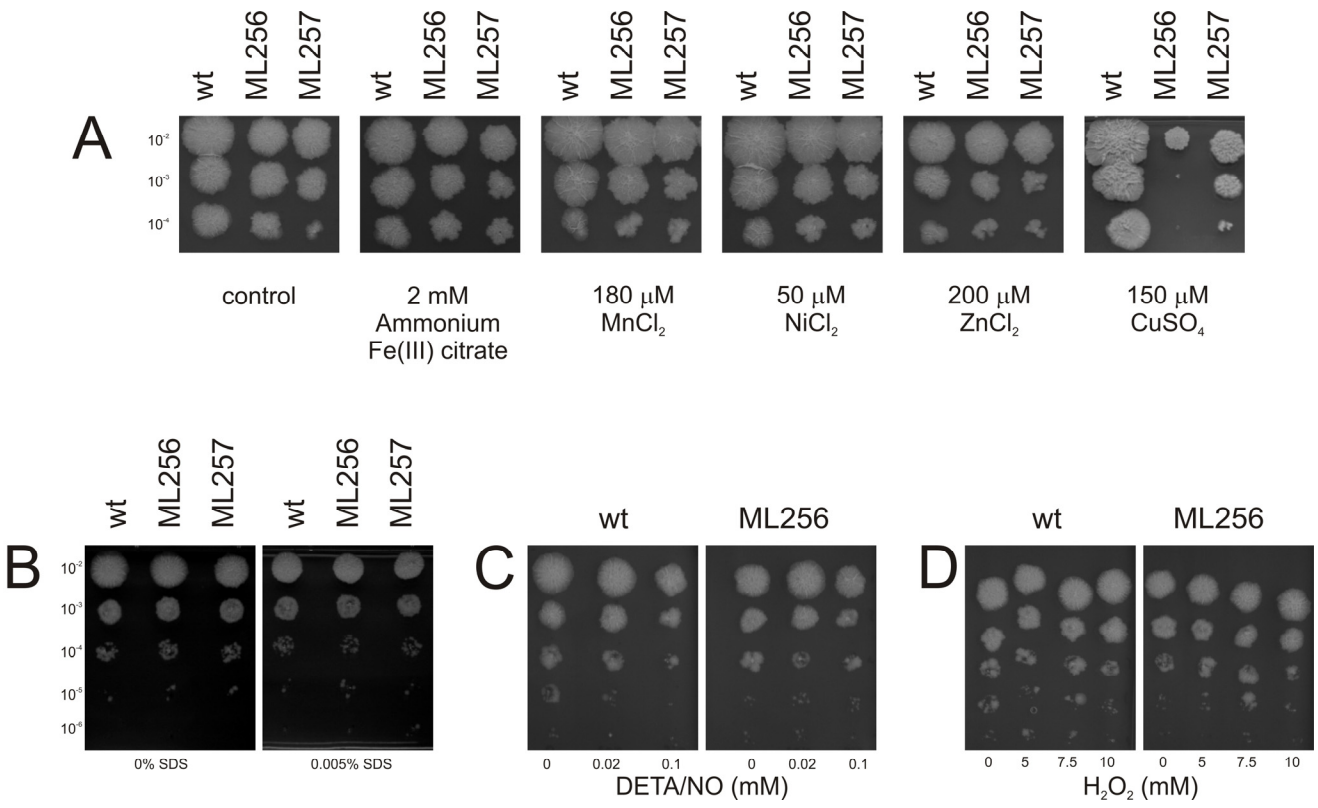

Fig. S9: Susceptibility of *Mtb* Δ*mctB* mutant (ML256) to metal ions, detergent, oxidative and nitrosative stress. Metal ions and SDS were added to 7H11 medium at the indicated concentrations. DETA/NO and H_2O_2 were added to liquid cultures as described in materials and methods. Cultures were adjusted to an OD_{600} of 1 and serially diluted to an OD of 10^{-6}. Of each indicated dilution, 5 μl were spotted on the plates which then were incubated at 37 ºC for 20 d (ammonium ferric citrate, $MnCl_2$, $NiCl_2$, $ZnCl_2$, H_2O_2, DETA/NO), 12 d (SDS) or 40 d ($CuSO_4$). Only the plates with the highest metal ion or SDS concentrations, at which we observed growth, are shown. A growth defect of ML256 was only observed at 150 μM $CuSO_4$, which was partially rescued by low level expression of $mctB_{Mtb}$ (A, last panel). No difference was observed in susceptibility of both strains to SDS (B), to nitrosative (C), or oxidative (D) stress.

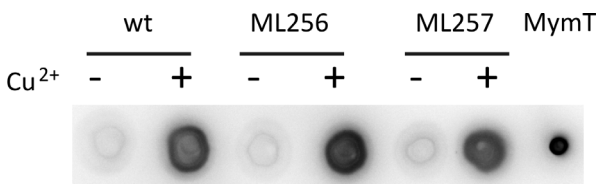

Fig. S10: Induction of *mymT* expression by copper is independent of the presence of MctB.

100 µM $CuSO_4$ was added to the culture. An equal amount of cells (based on OD_{600}) of *Mtb* WT, ML256 (Δ*mctB*) and the $mctB_{Mtb}$ complemented strain ML257 were harvested 24 h later, washed twice in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and then resuspended in protein sample buffer. Cells were lysed in a cup sonicator and boiled for 30 min at 100 ºC to extract the proteins and kill *M. tuberculosis*. Cell debris was removed by centrifugation. Equal volumes of the lysate (5 µl) were spotted onto a PVDF membrane and probed with anti-MymT antibody. Copper stress induced *mymT* expression in all three strains. Purified recombinant MymT peptide was used as a positive control.

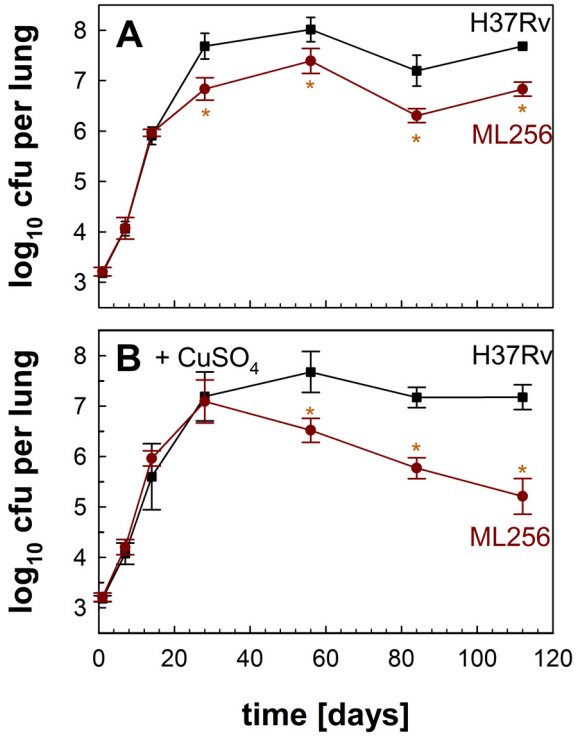

Figure S11. MctB-mediated copper resistance is required for full virulence of *M. tuberculosis* in mice.

BALB/c mice were infected with aerosols of wild-type *Mtb* H37Rv (black) and the *mctB* mutant ML256 (red). The colony forming units (cfu) were determined by plating lung homogenates from four mice. Error bars denote standard deviations. The bacillary loads that differ significantly from those of WT *Mtb* are denoted by an asterisk near the data point ($p < 0.01$ for all points as determined by the independent Student's t-test). **A.** Cfu counts in the lungs of infected mice. No $CuSO_4$ was added to the drinking water of mice. **B.** Effect of 118 mg/L $CuSO_4$ in the drinking water.

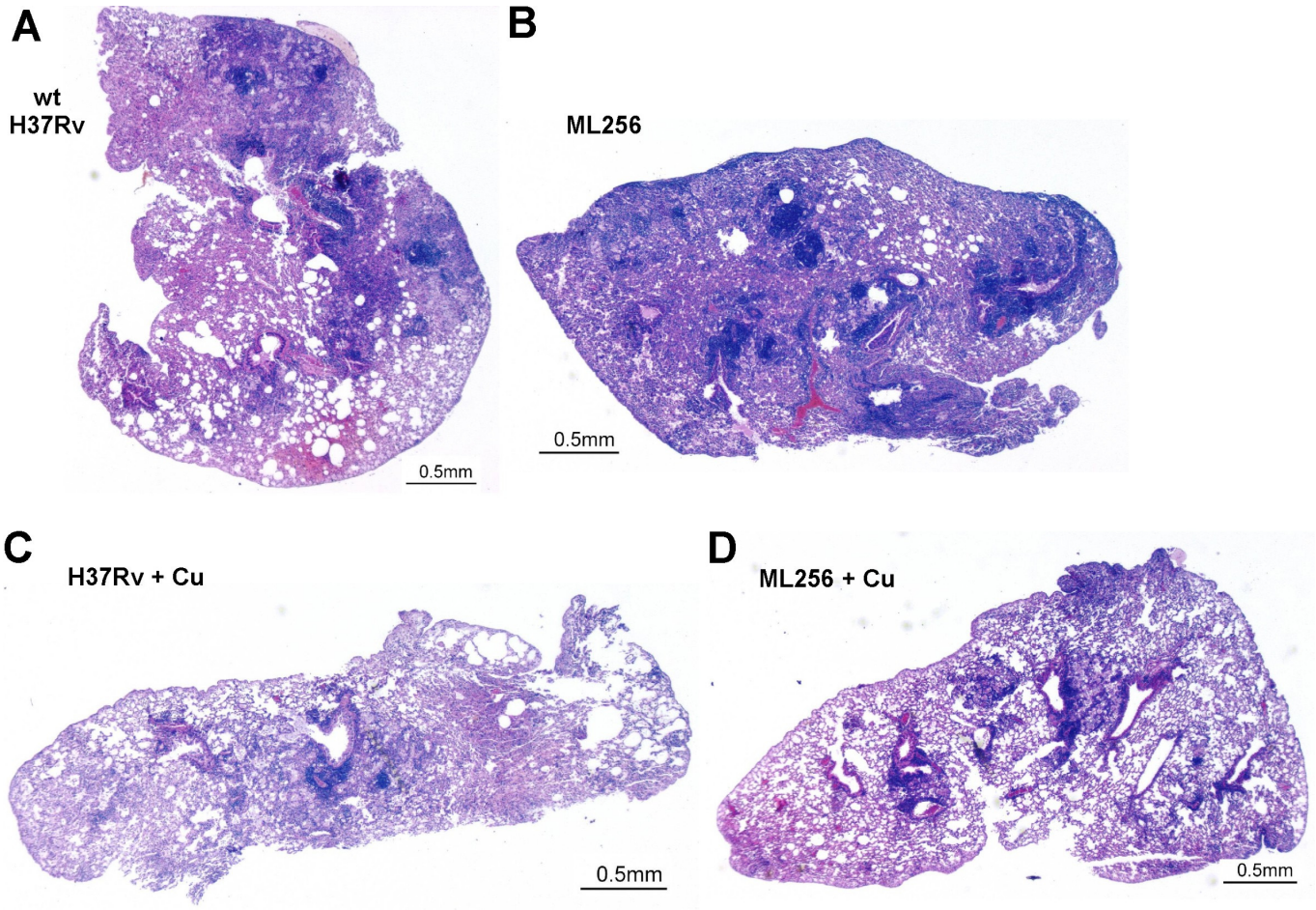

Fig. S12: Effect of copper on the pathology of mouse lungs infected with *M. tuberculosis* and the *mctB* mutant.

Lung sections of Balb/c mice infected with WT *Mtb* H37Rv (**A, C**) and the *mctB* mutant ML256 (**B, D**) were prepared 16 weeks after infection and stained with hematoxylin and eosin. Sections A and B were from mice kept under standard conditions, while sections C and D were from mice fed with additional 118 mg/mL copper sulfate. Pictures were taken with Nikon Eclipse E800 microscope outfitted with a Nikon DXM1200 digital camera (magnification: 20x).

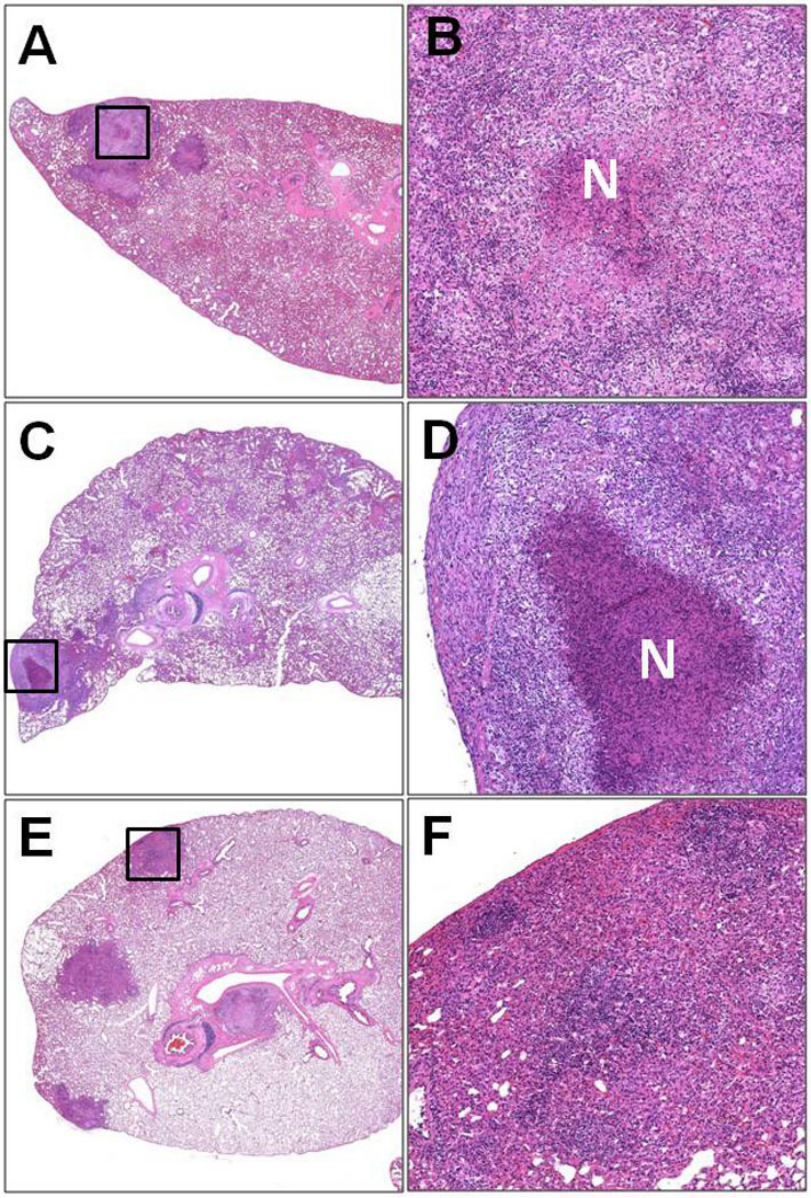

Fig. S13: Deletion of the *mctB* gene from *Mtb* H37Rv decreases lesions in the lungs of guinea pigs infected by low dose aerosol. C) The ML 256 strain of *Mtb* results in a significant reduction in lung lesion burden. One of five guinea pigs infected with ML256 retained the ability to induce primary lesion necrosis (D) but the overall average lesion burden for all animals within the treatment group was significantly reduced compared to the complemented strain (E and F) and the wild type (A and B). The complemented (E and F) and the wild type strain of *Mtb* both had increased lesion burden with characteristic foci of mixed inflammation that form discrete granulomas (A) that often have foci of central necrosis (N). Hematoxylin and eosin stain. A, C, E magnification 1.3X, B, D, F, magnification 10X.

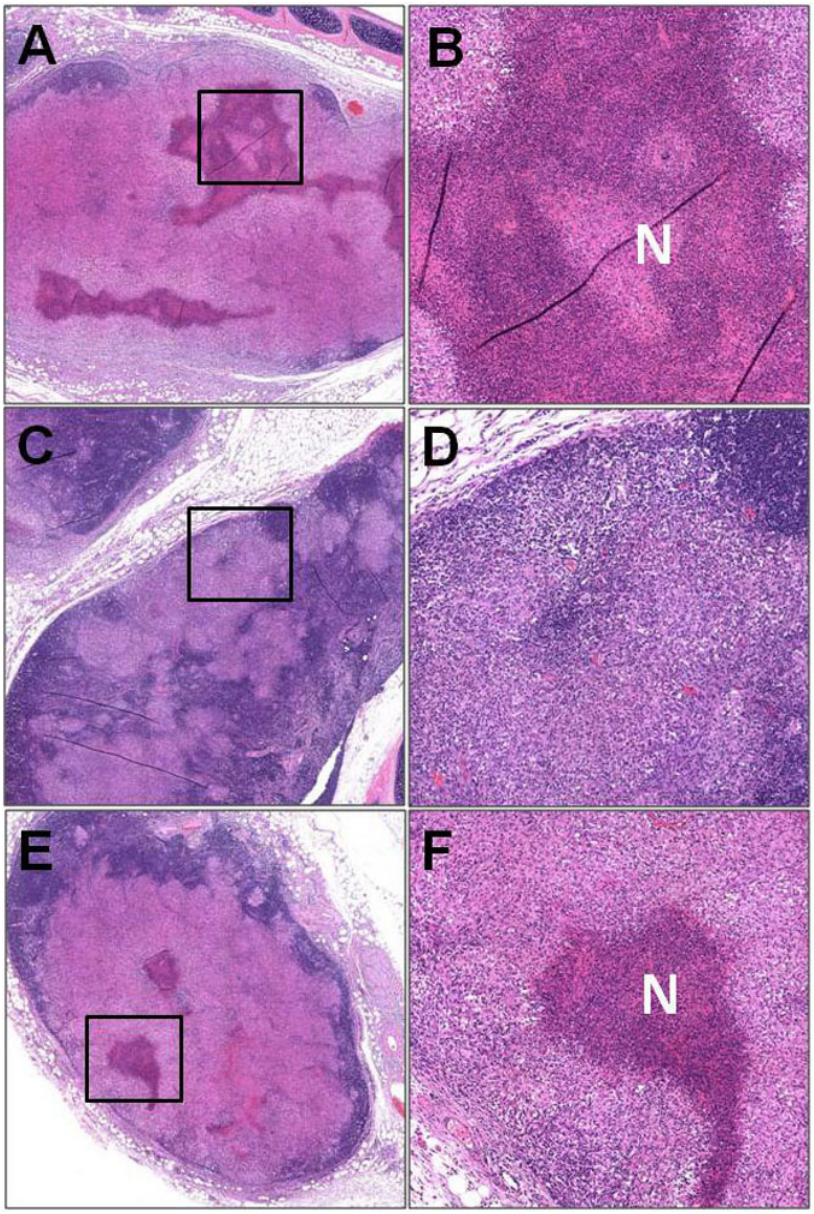

Fig. S14: Deletion of the *mctB* gene from *Mtb* H37Rv decreases extra-pulmonary lesions in the lymph nodes of guinea pigs infected by low dose aerosol. C) The ML 256 strain of *Mtb* results in a significant reduction in lymph node lesion burden. All 5 out of 5 guinea pigs infected with ML256 retained the ability to induce granulomatous lesions within the draining lymph nodes but few had lesion necrosis or completely effaced the lymph node architecture compared to the complemented strain (E and F) and the wild type (A and B). The complemented (E and F) and the wild type strain of *Mtb* (A and B) both developed severe lymph node involvement with characteristic foci of lesion necrosis (N) that was intermediate in the complemented strain (E) and more extensive in the wildtype strain (B). Hematoxylin and eosin stain. A, C, E magnification 2.5X, B, D, F, magnification 10X.

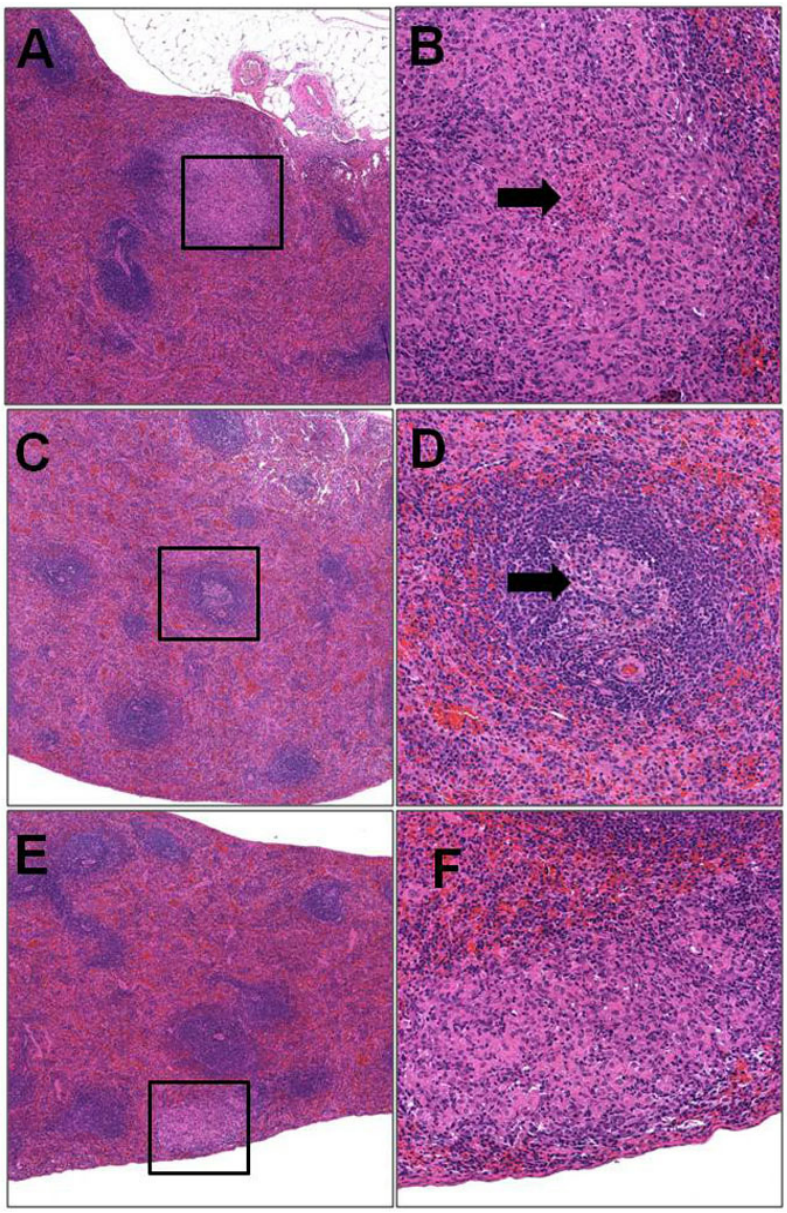

Fig. S15: Deletion of the *mctB* gene from *Mtb* H37Rv decreases extra-pulmonary lesions in the spleens of guinea pigs infected by low dose aerosol. C) The ML 256 strain of *Mtb* results in a significant reduction in spleen lesion burden. Guinea pigs infected with ML256 had few early (C and D) or no granulomatous lesions within the spleen by day 30 of infection. The complemented (E and F) and the wild type strain of *Mtb* (A and B) both developed splenic lesions that were intermediate in the complemented strain and usually lacked necrosis (F). In contrast, the wildtype strain induced a moderate splenic lesion burden (A) with early evidence of central lesion necrosis (B, arrow). Hematoxylin and eosin stain. A, C, E: magnification 5x; B, D, F: magnification 20x.

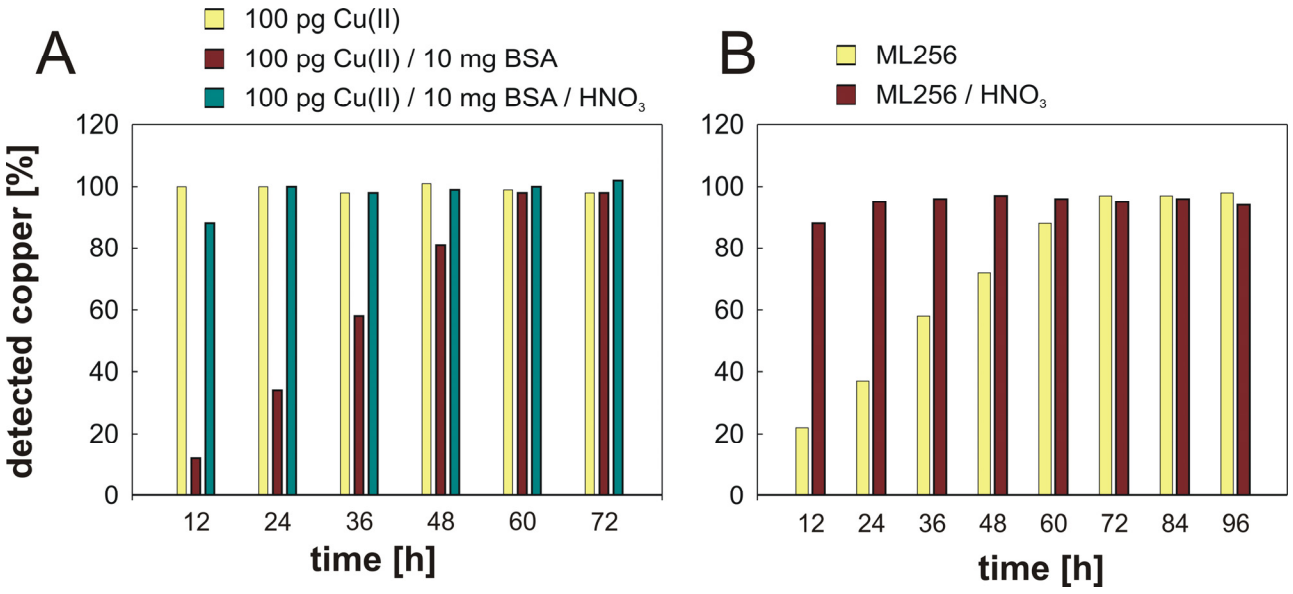

Fig. S16: Detection of copper in *M. tuberculosis* by dithizone.

Dithizone was used to detect copper in cellular samples. **(A)** In order to validate our results we tested the accuracy of the assay conditions by mixing 100 pg $CuSO_4$ with 10 mg BSA, a copper binding protein. Nitric acid (HNO_3) was added to accelerate the release of copper bound to proteins. Nearly 100 % of the copper input was detected after 60 h. Addition of HNO_3 decreased the incubation time significantly to 24 h but gave similar results. **(B)** We also determined the copper content of the *M. tuberculosis mctB* mutant ML256 that had been grown in the presence of 25 μM Cu(II). HNO_3-treatment enabled detection of Cu(II) by dithizone within 24 h while it took up to 72 h for the untreated sample.

SUPPLEMENTARY TABLES

Oligonucleotide	Sequence (5'→3')
max_red1_FW	CTAGTTTAAACCAAGAAGATCCTTTGATATTTTCTACGGGGTCTGACGCTCAGTCGAGTATACGAGGCGCGCCA GATTTAAATG
max_red1_RW	GATCCATTTAAATCTGGCGCGCCTCGTATACTCGACTGAGCGTCAGACCCCGTAGAAAATATCAAAGGATCTTC TTGGTTTAAA
max_red2_FW	AATTCGCTAGCACATGCATGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATGTTTAAAC
max_red2_RW	TCGAGTTTAAACATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCATGCATGTGCTAGCG
max_loxP1_FW	GATCCCGATTTAAATAACTTCGTATAATGTATGCTATACGAAGTTATCCCGGGCTGCA
max_loxP1_RW	GCCCGGGATAACTTCGTATAGCATACATTATACGAAGTTATTTAAATCGG
max_loxP2_FW	GATCCCGGGATACGCGTATAACTTCGTATAATGTATGCTATACGAAGTTATTAATTAAAGCTAGCG
max_loxP2_RW	AATTCGCTAGCTTTAATTAATAACTTCGTATAGCATACATTATACGAAGTTATACGCGTATCCCGG
[1]mpoS_do_FW	CCTTAATTAACACCCTCACCGACGACAAGAAC
[2]mpoS_do_RW	GCATGCATGTTTCGTGGCGGTTTGCGGGTG
[3]mpoS_up_FW	CTATGGCGCGCCGTGGTCAATGCCTCGCCTTCC
[4]mpoS_up_RW	GAGGATTTAAATCACGCAGACCCGACAGCACG
[1]mpoS_Sdopt_1	CGTTAATTAAGCAGAAAGGAGGTTAATCTATGATAACGCTACGGGCGCACGCGATC
CN842	GTGTAACCCACGAGCCGTCAAC
CN991	GCCACGCTCTACCGCA

Table S1. Oligonucleotides used in this work. Restriction sites used for cloning are underlined. [1]PacI, [2]BfrBI, [3]AscI, [4]SwaI.

Plasmid	Parent vector, relevant genotype and properties	Source or reference
pCreSacB1	p_{groEL}-*Cre*, oriE, oriM, *sacR*, *sacB*, *aph;* 7891 bp	Dr. Steyn
pBluescript KS+	*bla*, pUC-origin, f1(+), *lacZ*'; 2958 bp	Stratagene
pBS346	*bla,* ColE1 origin, *loxP-hyg-sacB-sacR-loxP*; 5725 bp	Dr. Steyn
pMS2	ColE1 origin, *hyg*, oriM; 5229 bp	(1)
pMN016	ColE1 origin, *hyg*, oriM, p_{smyc}-*mspA;* 6164 bp	(2)
pMN035	ColE1 origin, *hyg*, oriM, p_{smyc}-$mctB_{Mtb}$; 6484 bp	(3)
pML113	FRT-*hyg*-FRT, *bla*, $attP_{L5}$, ColE1 origin; 4365 bp	(4)
pML343	*bla*, pUC origin, f1(+), up_hom, *loxP-hyg-loxP*, down_hom, *sacR*, *sacB*	this study
pML451	ColE1 origin, *hyg*, oriM, p_{smyc}-$mctB_{Ms}$; 6469 bp	this study
pMN437	ColE1 origin, *hyg*, oriM, p_{smyc} gfp_m^{2+}, 6236 bp	(5)
pCG63	pAL5000ts, 8977 bp	(6)
pML955	ColE1 origin, *hyg, int*, p_{imyc}-$mctB_{Mtb}$, $attP_{L5}$, 6143 bp	this study
pML515	pUC origin, pAL5000ts, *sacR*, *sacB, xylE,* up_hom, *loxP*-p_{imyc}-*mycgfp2+-hyg-loxP*, down_hom, 11859 bp	this study

Table S2: Plasmids used in this work. Up- and downstream homologous sequences of the *mctB* genes are subscripted as up_hom and down_hom in pML343 for *M. smegmatis* and pML515 for *M. tuberculosis*. "Origin" means origin of replication. The genes *bla, hyg* and *aph* confer resistance to ampicillin, hygromycin and kanamycin, respectively. The $attP_{L5}$ site is required for site specific integration of plasmids into the chromosomal *attB* site by the mycobacteriophage L5 integrase gene, *int*. The site-specific recombinase Cre excises DNA fragments that are flanked by *loxP* recognition sites. pAL5000ts denotes the temperature-sensitive origin of replication (6) of the pAL5000 plasmid (7). The constitutive mycobacterial promoters p_{smyc} and p_{imyc} have been described previously (1, 8). The genes *ms3747 (M. smegmatiseg_3747)* of *M. smegmatis* and *rv1698* of *M. tuberculosis* are named $mctB_{Ms}$ and $mctB_{Mtb}$, respectively. The *sacS* gene of *B. subtilis* encodes the counterselective marker levansucrase that mediates sensitivity to sucrose (9). Its expression is regulated by SacR.

Strain	Parent strain and relevant genotype	Source or reference
E. coli DH5α	*recA1; endA1; gyrA96; thi; relA1; hsdR17(rK-;mK+); supE44; ϕ80ΔlacZΔM15; ΔlacZYA-argF; UE169*	(10)
M. smegmatis SMR5	mc^2155 derivative, Sm^R (*rpsL**)	(11)
M. smegmatis ML75	SMR5 derivative, *mctB*::pML343, Hyg^R	this study
M. smegmatis ML76	ML75 derivative, *mctB*::*loxP-hyg-loxP*, Hyg^R	this study
M. smegmatis ML77	ML76 derivative, *mctB*::*loxP*	this study
M. tuberculosis H37Rv	wild-type	ATCC# 25618
M. tuberculosis ML256	H37Rv derivative, *Δ$mctB_{Mtb}$ (rv1698*::*loxP*)	this study
M. tuberculosis ML257	ML256 derivative, *attB*::pML955; Hyg^R	this study

Table S3: Strains used in this work. The annotations Sm^R and Hyg^R indicate that the strain is resistant to the antibiotics streptomycin and hygromycin, respectively. It should be noted that all *M. smegmatis* strains are derivatives of SMR5 and are therefore resistant to streptomycin. This is not indicated in the table except for SMR5. The *rpsL* gene encodes a mutated ribosomal protein S12 (K43R) which confers streptomycin resistance (Sm^R).

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions.

All bacterial strains used in this study are listed in table S1. Unless mentioned otherwise, *M. smegmatis* strains were grown in Middlebrook 7H9 liquid medium supplemented with 0.2% glycerol, 0.05% Tween 80 or on Middlebrook 7H10 agar supplemented with 0.5% glycerol using premixed powders (Difco). Self-made Middlebrook medium (7H9sm or 7H10sm) was prepared according to the recipe of the manufacturer (Difco), except that $CuSO_4$ and Malachite Green were omitted. Middlebrook media were always supplemented with OADC (8.5 g/L NaCl, 20 g/L dextrose, 50 g/L bovine albumin (fraction V), 0.03 g/L catalase, 0.6 ml/L oleic acid) when used for *M. tuberculosis*. Hartmans de-Bond (HdB) medium was prepared as previously described (12). 7H10sm and HdB medium were solidified with Agar Noble (Difco). $CuSO_4$, ammonium iron (III) citrate, $MnCl_2$, $ZnCl_2$, $NiCl_2$, SDS, H_2O_2 and DETA/NO were added separately to achieve desired concentrations. *Escherichia coli* DH5α was used for all cloning experiments and was routinely grown in LB medium at 37 °C. The following antibiotics were used when required: ampicillin (100 µg ml^{-1} for *E. coli*), kanamycin (30 µg ml^{-1} for mycobacteria), hygromycin (200 µg ml^{-1} for *E. coli*, 50 µg ml^{-1} for mycobacteria).

Construction of plasmids.

The non-replicative suicide vector pML343 was used for deletion of *mctB* in *M. smegmatis*. Two identical *loxP* sites (loxP1, loxP2) and two linkers (link1, link2) were cloned separately into the vector pBluescript KS+ cut by the restriction endonucleases BamHI/PstI, BamHI/EcoRI, BamHI/SpeI and EcoRI/XhoI to give the four vectors pML311 to 314, respectively. DNA fragments containing the *loxP* sites (loxP1, loxP2) and the linkers (link1, link2) were generated by hybridizing 400 pmol of each of the complementary phosphorylated oligonucleotides (loxP1: max_loxP1_FW and max_loxP1_RW; loxP2: max_loxP2_FW and max_loxP2_RW; linker1: max_red1_FW and max_red1_RW; linker2: max_red2_FW; max_red2_RW; see table S1). Mixtures were incubated for 10 min at 100°C, for 4 h at 65°C and overnight at 37°C. Restriction sites needed for cloning resulted directly from the correct hybridization of complementary oligonucleotides. Next, the MluI/SmaI fragment of vector pBS346 containing the *hyg* gene was cloned into the vector pML312 digested with the same restriction

endonucleases to give pML315. The 3242 bp BamHI/NheI fragment of pML315 was then integrated into pML314. The resulting vector pML316 was cut with XhoI/SwaI and the *hyg* gene containing fragment was cloned into the backbone of pML311 similarly digested to give pML317. The resulting *loxP-hyg-loxP* cassette flanked by XhoI and SwaI sites was then cut out and cloned into the backbone of pML313 to create pML318. Using PCR, the restriction sites AscI and SwaI were attached to the upstream homolog region of *mct*B using the primer mpoS_up_FW and mpoS_up_RW, while BfrBI and PacI were introduced to the downstream region (mpoS_do_FW and mpoS_up_RW). Individual sequences were cloned to bracket the *loxP-hyg-loxP* cassette in pML318 via the corresponding restriction sites. Since the *hyg* cassette from pBS346 mediated only a weak resistance to hygromycin, the *hyg* cassette was replaced by that of pML113 and the *sacB* cassette from pBS346 was introduced in front of the upstream homolog region to generate the $mctB_{Ms}$ deletion vector pML343. For deletion of *rv1698* of *Mtb*, the thermo-sensitive pAL5000ts origin of replication from pCG63 (6), was inserted to uncouple recombination from the transformation efficiency. The upstream and downstream regions of *ms3747* were replaced with those of *rv1698*. To improve the screening for single and double crossover candidates, reporter genes were introduced. The *mycgfp2+* originating from pMN437 was put upstream of the *hyg* gene to create a *loxP-mycgfp2+-hyg-loxP* cassette. The *xylE* gene from pXylE4 was integrated into the plasmid backbone. The final $mctB_{Mtb}$ deletion vector was called pML515. All plasmids were verified by DNA sequencing.

Southern Blots.

Chromosomal DNA was isolated from *M. smegmatis* as described previously (13). For Southern blot analysis, 5 μg of chromosomal DNA was digested with 20U PstI overnight at 37°C, separated on a 1% agarose gel and transferred in 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) to a positively charged nylon membrane (PVDF, Amersham). The downstream region of $mctB_{Ms}$ from pML343 was cut out by BfrBI and PacI and used to generate the *mctB* probe using the Dig DNA labelling and detection kit (Roche). All other steps were carried out as described previously (14, 15).

Construction of the Δ*mctB* mutant of *M. smegmatis*.

M. smegmatis SMR5 was transformed with 4 μg of pML343, plated on Middlebrook 7H10/hygromycin medium and incubated at 37°C. After four days, two colonies grew. Chromosomal DNA was isolated and Southern blot experiments were done as described above, confirming that a single cross-over took place in both candidates. The deletion plasmid pML343 was inserted in the downstream homolog region of candidate two which was named ML75. *M. smegmatis* ML75 was grown in Middlebrook 7H9/hygromycin broth, filtrated through a 5 μm-filter and plated on Middlebrook 7H10/hygromycin plates containing 10% sucrose to select for double cross-overs (DCO). Approximately 40% of all clones obtained from ML75 grew as small colonies clearly distinguishable from bigger colonies after five days of incubation at 37°C. The DCO event was confirmed for small colonies. A verified DCO candidate was named *M. smegmatis* ML76 and transformed with pCreSacB1 to excise the *loxP* flanked *hyg* cassette. A colony was picked from Middlebrook 7H10/kanamycin plates and grown in Middlebrook 7H9/kanamycin broth for 24 h to excise the *hyg* cassette. The culture was filtered through a 5 μm filter and plated on Middlebrook 7H10 plates containing 10% sucrose to counter-select for pCreSacB1. Ten colonies were picked and grown on Middlebrook 7H10 plates containing either hygromycin, kanamycin or no antibiotic. Susceptibility to both antibiotics indicated the loss of both the *hyg* cassette and the pCreSacB1 vector. The excision of the *hyg* cassette was confirmed by Southern blot. One of the positive clones was named ML77. Sequencing of a PCR fragment amplified of the *mctB* region from chromosomal DNA of ML77 confirmed that 29 bp located 116 bp downstream of the gene start were replaced by the *loxP*-site. This introduced stop codons in all three reading and thereby inactivates the *mctB* gene.

Construction of the Δ*mctB* mutant of *M. tuberculosis*.

The *rv1698* deletion vector pML515 which carries the reporter genes gfp_m^{2+} and *xylE*, was transformed into *Mtb* H37Rv cells. After 21 days at 37°C, fluorescent colonies that also turned yellow in the presence of 1% catechol were picked and transferred in 10 ml of Middlebrook 7H9 broth supplemented with hygromycin. The culture was grown at 37°C to an OD_{600} exceeding 1.0. Then, 100 µl were plated on Middlebrook 7H10 medium containing hygromycin and incubated at 40°C to

integrate the plasmid by homologue recombination. A colony tested positive for both reporters was picked and grown in Middlebrook 7H9/hygromycin broth. Appropriate volumes of the culture with an OD_{600} exceeding 1.0 were plated on Middlebrook 7H10/hygromycin medium supplemented with 2% sucrose to select for double crossover. The loss of *xylE* expression indicated the excision of the plasmid backbone leaving the *loxP- gfp_m^{2+}-hyg-loxP* cassette. After 30 days at 37°C, four fluorescent colonies without XylE activity were picked and transferred into Middlebrook 7H9/hygromycin broth. Chromosomal DNA was isolated and the destruction of the gene was verified by PCR. One candidate was picked and competent cells were transformed with pCreSacB1 to excise the *loxP- gfp_m^{2+}-hyg-loxP* cassette. One colony was picked and grown in Middlebrook 7H9/kanamycin broth. Appropriate dilutions were plated on Middlebrook 7H10 medium supplemented with 2% sucrose and incubated at 37°C. After 21 days four non-fluorescent colonies were picked and grown in Middlebrook 7H9 broth. After the culture reached an OD_{600} of approximately 1, 5-µl drops of the culture were dropped on Middlebrook 7H10 plates containing either kanamycin, hygromycin or no antibiotics. All colonies were sensitive to both antibiotics indicating that the *hyg* cassette had been efficiently excised and pCreSacB1 was lost. Sequencing of a PCR fragment obtained from chromosomal DNA confirmed that 39 bp located 112 bp downstream of the *mctB* gene start were replaced by the *loxP* site. One of the positive clones was named ML256. For complementation, competent cells of ML256 were transformed with the integrative plasmid pML955 carrying *mctB* under the control of the p_{imyc} promoter. The plasmid integrates at the *attB* site of mycobacteriophage L5. The complemented mutant was named ML257. ML256 was also complemented with the replicative *mctB* expression plasmid pMN035. Absence of *mctB* in ML256 and the expression of *mctB* in the complemented strains ML257 and ML256 carrying the plasmid pMN035 was analyzed and confirmed by Western blot using the monoclonal antibody 5D1.23.

Copper accumulation.

The copper content of mycobacteria was analyzed using the photometric dithizone assay. 300 µl of cell suspension were mixed with 300 µl fresh dithizone $5x10^{-4}$ M (dissolved in $CHCl_3$) and 2.7 ml $CHCl_3$. The two-phase system was left to react for up to 120 h. The absorption maximum at 553 nm (ε

= 11,709 M^{-1} cm^{-1}) is characteristic for the Cu^{2+}-dithizone complex and was measured twice a day. A calibration curve with known $CuSO_4$ concentrations showed that the absorption at 553 nm linearly depends on the concentration of Cu(II) from 10^{-9} M to 10^{-2} M Cu(II). The presence of bovine serum albumin (BSA), a protein that binds copper, does not interfere with the sensitivity of the assay. However, a longer incubation period of at least 60 h is required (fig. S16A). Addition of HNO_3 clearly accelerated detection of copper in the presence of BSA (fig. S16A) and cellular samples (fig. S16B), but the amount of detected copper at the respective endpoints of the reaction was identical. Samples needed to be concentrated or diluted to measure Cu(II) concentrations smaller than 10^{-7} M or larger than 10^{-4} M, respectively. A calibration curve was used to determine the copper concentrations in mycobacterial cells. The concentration of copper traces in self-made 7H9 medium was less than 1 μM.

Preparation and analysis of protein extracts.

M. smegmatis was streaked on 7H10 plates (7H10sm) without copper and grown for 5 days at 37°C while sealed in a zip-top bag. A pre-culture of 20 ml self-made 7H9 medium (7H9sm) was inoculated and grown over night. On the next day, 7H10sm medium with or without copper was inoculated to an OD_{600} of 0.05 and shaken at 37°C. Unless otherwise noted, the culture was centrifuged and 1 g of wet cells was resuspended in 6 ml PBS (140 mM NaCl, 2 mM KCl, 10 mM K_2HPO_4/KH_2PO_4, pH 7.4) and lysed by sonication. After centrifugation at 9000xg the pellet was washed in 6 ml PBS, sonicated again and centrifuged. The final pellet was resuspended in 1 ml PBS containing 2% SDS and incubated at 100°C for 15 min. After centrifugation at 16,000 g, the protein content of the supernatant was determined by using bicinchoninic acid (BCA Protein Assay Kit, Pierce).

To extract MctB from *M. tuberculosis*, cells were grown in Middlebrook 7H9 medium. 15 ml of culture with an OD_{600} of 1.0 were centrifuged at 3000 g. The pellet was resuspended in 250 µl of 4x loading buffer containing 2% SDS and boiled for 30 min to extract proteins and kill *Mtb*. The boiled samples were centrifuged to remove debris before loading them onto the gel. 40 µl of each sample was analysed in Western blots as described (2).

For *M. smegmatis*, 50 μg protein of each sample was loaded onto a denaturing 10% polyacrylamide gel. The protein gel was blotted for four hours at 150 mA in transfer buffer (25 mM Tris base, 192 mM

Glycine, 0.1% SDS, 20% methanol) onto a polyvinylidene difluoride (PVDF) membrane. MctB protein was detected by the monoclonal antibody 5D1.23 that detects MctB proteins from both *M. smegmatis* and *Mtb*. The anti-MymT antibody and the purified recombinant MymT protein were kindly provided by Dr. Ben Gold (Weill Cornell Medical College, New York). Horseradish peroxidase-coupled anti-mouse or anti-rabbit antibodies were used for detection in combination with the ECL plus kit (Amersham). A chemoluminescence imaging system and the software LabWorks (UVP) were used to visualize and quantify the luminescence.

Generation of monoclonal antibodies against MctB.

Hybridomas were generated from B-cells that were obtained from mice immunized with recombinant *M. tuberculosis* MctB protein purified from *E. coli*. The supernatant of hybridomas was examined in ELISA experiments for binding to 1 ng recombinant MctB protein purified from *E. coli*. Positive hybridomas were subcloned and tested again. The specificity of 23 selected antibodies was examined by Western blots using membrane extracts from *M. smegmatis mctB* mutant ML77 and its complemented strains overexpressing MctB from both *M. tuberculosis* and *M. smegmatis.* The IgG monoclonal antibody 5D1.23 was classified as an IgG antibody and recognized MctB from both organisms. A 1:100 dilution of the hybridoma supernatant was used for detection of MctB in Western blot experiments.

Role of MctB in virulence of *M. tuberculosis* in mice.

A preliminary copper tolerance study was performed, in which three groups of BALB/c mice (Charles River) were given water *ad libitum* containing either 118 mg/L, 1180 mg/L, or 11.8 g/L copper sulfate pentahydrate (Sigma). The mice were monitored for two weeks. A concentration of 118 mg/L copper sulfate pentahydrate did not cause any signs of distress in mice and was chosen for further experiments.

Mid-log phase cultures of *Mtb* WT (H37Rv), the *mctB* mutant ML256 and the complemented mutant ML257 were diluted to OD_{600} ~ 0.08 to implant 500-1000 bacilli in the lungs of mice by using a Middlebrook inhalation exposure system (Glas-Col). Eighty 6-week old BALB/c mice were infected

with WT *Mtb* or ML256, and 40 mice were infected with ML257. One day following infection, half of the mice infected with WT *Mtb* or ML256 were separated and given water *ad libitum* containing 118 mg/L copper sulfate pentahydrate. Four mice from each group were weighed and sacrificed at days 1, 7, 14, 28, 84, and 112 post-infection in order to determine the number of bacilli in the lung and spleen. Mouse organs were aseptically removed, homogenized by bead-beating, and serially diluted. Appropriate dilutions were plated onto Middlebrook 7H11 agar plates to determine the colony forming units. For histological analysis, representative tissue samples from each group were fixed in 10% formaldehyde, embedded in parraffin, sectioned, and stained with hematoxylin and eosin using standard procedures.

Role of MctB in virulence of *M. tuberculosis* in guinea pigs.

Guinea pigs. Specific-pathogen free, female Hartley guinea pigs (weight, ~400 g) were purchased from Charles River Laboratories (North Wilmington, MA). After a two week acclimation period, 15 animals were marked with microchips for identification and randomly assigned to three separate groups of five animals. Two animals were housed per cage in a biosafety level 3 animal laboratory with free choice food and water. All procedures and experimental protocols were approved by the Colorado State University Biosafety and Animal Care and Usage Committees.

Experimental infections in guinea pigs. Guinea pigs were infected with *Mycobacterium tuberculosis* strain H37Rv, ML 256 and ML257 using a Madison chamber aerosol generation device, calibrated to deliver approximately 20 bacilli via the airways. Thirty days after infection, animals were heavily sedated with an intramuscular injection of xylazine and ketamine and then euthanized with an intraperitoneal barbiturate overdose (120 mg/kg). At necropsy lungs, draining mediastinal lymph nodes and spleen were collected for histopathology and bacterial load determinations. The tissue bacterial load was determined by plating serial dilutions of tissue homogenates on nutrient Middlebrook 7H11 agar. The CFU were counted after 3 weeks of incubation at 37°C. The bacterial load for each organ was calculated and converted to logarithmic units. The data were expressed as the mean $\log_{10}$ number of CFU/mL plus/minus the standard deviation for each group.

Histological analysis. Representative samples of lung (right caudal lobe), spleen, and lymph node from each *Mtb* infected guinea pig was fixed for a minimum of 48 hours in 4% paraformaldehyde in PBS then transferred to 70% ethanol. Tissue sections were embedded in paraffin wax and cut to 5 μm on a microtome, mounted on glass slides, deparaffinized in xlylene and stained with hematoxylin-eosin following graded ethanol washes. The lesion burden for lung, spleen and lymph node was quantified using a stereology-based method referred to as the area fraction fractionator with the investigator blind to all treatment groups. The stereology workstation consisted of a Nikon 80i research microscope equipped for bright-field and fluorescence microscopy a three-axis computer-controlled stepping stage with linear grid encoders, a *z*-axis motorized specimen stage for automated sampling all integrated by a personal computer using StereoInvestigator software version 9.10.2 (MBF Bioscience, Williston, VT).

The lesion burden for each tissue section was estimated from the area of normal tissue replaced by characteristic granulomatous inflammation, necrosis and dystrophic calcification. Representative lung, spleen, and lymph node tissue sections were evaluated at 20x, 40x and 100x magnification, respectfully. A total of 10 to 15 fields were randomly selected by the computer and the total tissue and lesion areas estimated using a counting frame (2,000 μm^2, 1,000 μm^2, 500 μm^2) containing probe points with a grid spacing of 200 μm, 100 μm, 50 μm respectively to define the areas of interest. The data are expressed as the mean percentage of normal lung, spleen or lymph node replaced by lesions within a treatment group. The lesions represented by the photomicrograph are from the tissue sections that are the closest to the mean value for each group.

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