Supporting Information

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SI Materials and Methods

Bacterial Strains, Media, and Reagents. Mycobacteria were cultured in Middlebrook 7H9 medium containing 0.05% Tween-80 and 10% albumin-dextrose-catalase (ADC) in the presence of kanamycin and hygromycin as needed. Chelated Sauton's medium consisted of 60 mL of glycerol, 0.5 g of KH_2PO_4, 2.2 g of citric acid monohydrate, 4 g of asparagine, and 0.5% Tween-80. After adjustment of the pH to 7.4, the medium was stirred 1–2 days at room temperature with 10 g of Chelex 100 resin (Sigma). The medium was filtered, and 1 g of $MgSO_4 \cdot 7H_2O$ was added as a sterile solution.

Mycobacterial Mutant Construction. To generate a *Mycobacterium bovis* BCG conditional *esx-3* mutant, the 5′ end (1000 bp) of *mb0290* (forward 5′-ggccttaattaagaaggagatatacatATGGCGGGCGTAGGTGAAGG-3′, reverse 5′-actggatatcGCAGCGCGGCCTTGCGTTCG-3′) was amplified from *M. bovis* BCG genomic DNA and cloned into the suicide vector pSES [a derivative of pSE100 (1) created in this laboratory] downstream of the tetracycline-inducible promoter and confirmed by sequencing. Wild-type *M. bovis* BCG first was transformed with pMC1s containing tetR (1) and selected on 7H10 containing kanamycin. This strain then was transformed with pSES-*0290*, and transformants were selected on 7H10 plates containing kanamycin, hygromycin, and varying concentrations of anhydrotetracycline (ATC) (0–50 ng/mL). Single crosses at the *esx-3* locus were obtained only on plates with very low concentrations of ATC and were confirmed by PCR using primers specific to the tetracycline promoter (5′-GCACGGCATACATCATTTCGACGCCG-3′) and *mb0290* gene (5′-TCATGCCCGCACCGAGAG-3′).

To construct the *M. bovis* BCG and *M. tuberculosis* deletion mutants, 1-kb flanking regions 5′ of *mb0290* or *rv0282* and 3′ of *mb0301* or *rv0292* (for *esx-3* deletion) and 5′ and 3′ of *mb0291* or *rv0283* (for *mb0291* and *rv0283* deletions) were amplified from *M. bovis* BCG or *M. tuberculosis* genomic DNA and cloned into suicide vector pJM-1 containing a hygromycin–chloramphenicol resistance cassette and *sacB*. *M. bovis* BCG and *M. tuberculosis* transformants were screened by standard microbiological methods. The PCR with primers outside (*rv0280*, 5′-CCTGGAGATCGGGGCTGA-3′) and inside each flank (5′-AGTGAACGTCCCTGACA-3′) were used to confirm correct single crossover events. We did not detect single crosses at *mb0291* or *rv0283*. Confirmed *esx-3* single crosses were screened by standard methods and by PCR using primers specific to *rv0280* and *rv0294* (GACGTCGGGATCCCACAT). No double crossover events were detected from the *esx-3* single crosses.

To create the *M. smegmatis esx-3* deletion mutant, regions flanking 1 kb 5′ to *msmeg0614* and 3′ to *msmeg0624* were amplified from *M. smegmatis* genomic DNA, stitched together by PCR, and cloned into suicide vector pJM-1 containing a hygromycin–chloramphenicol resistance cassette and the counterselectable marker *sacB*. *M. smegmatis* transformants were screened by standard methods and by PCR using primers specific to the flanks (forward, 5′-ACTGGAATCATATGCAAAGCGACCTTGGATGAC-3′; reverse, 5′-AGGTAGACCATATGGGTGGTCGGTTCCTTTCTC-3′) and to *msmeg0622*, a gene within the putative deletion. Candidates were confirmed by PCR using primers outside the flanks and to multiple genes within the putative deletion.

Similar procedures were followed to construct the unmarked, in-frame *M. smegmatis msmeg0622* deletion: 1-kb regions 5′ and 3′ of the gene were cloned into the pJM-1 suicide vector, and transformants were selected on hygromycin and then counterselected on sucrose. Candidate gene deletions were screened by PCR using primers both within the gene and outside of the flanks used to create the knockout vector. The complementing construct was created by amplifying *msmeg0622* from genomic *M. smegmatis* DNA and cloning it into pJEB402, an integrating plasmid that contains a kanamycin resistance cassette.

To construct the *M. smegmatis* insertional mutants, 1 kb within the *fxbA* and *mbtD* genes was amplified from *M. smegmatis* genomic DNA and cloned into suicide vectors pSES, containing a hygromycin resistance cassette, and p402SK, containing a kanamycin resistance cassette, respectively. *M. smegmatis* transformants were screened by standard methods, and siderophore production was assessed for candidate mutants.

Immunoblotting. Wild-type and Δ*esx-3 M. smegmatis* transformed with pTetG-*esxGH*-c-myc were inoculated from frozen stocks into 7H9 medium, grown to saturation, and then diluted 1:500 in chelated Sauton's medium with appropriate antibiotics in the presence or absence of 12.5 μM $FeCl_3$. Bacteria were grown to OD_{600} of 0.4–0.5, centrifuged, resuspended in chelated Sauton's medium containing 10 ng/mL ATC in the presence or absence of 12.5 μM $FeCl_3$, and grown an additional 5–6 h.

M. bovis BCG transformed with pMV762-*esxG*-6Xhis-*esxH*-c-myc were cultured in 7H9 medium containing iron to OD_{600} of 0.5–0.6, washed once in Sauton's medium, and further incubated for 24 h in either low or high iron Sauton's medium.

Proteins from cell pellets and supernatants were run on 10–20% Tris-Tricine gels (Invitrogen) and revealed using an anti-c-myc antibody (Novus Biologicals). An antibody to ClpP1P2, an intracellular protein, served as a loading and lysis control (kind gift of Alfred L. Goldberg).

RNA Extraction and Quantitative RT-PCR. Total RNA was extracted from *M. smegmatis* or *M. bovis* BCG log-phase cultures, purified using a RNA miniprep kit (Qiagen), and treated with DNase I (amplification grade; Invitrogen). Approximately 0.2–0.5 μg of RNA was used for cDNA synthesis, using the SuperScript III First-Strand Synthesis System (Invitrogen) per the manufacturer's protocols. Approximately 1–2 μL of the cDNA was used for quantitative, real-time RT-PCR. Quantitative RT-PCR was performed using SYBR Green per standard protocols. The PCR primers used are shown in Table S2. The value of $2^{-\Delta\Delta Ct}$ was calculated to represent the amounts of RNA relative to the amounts of *sigA* for each gene.

Mass Spectrometry. Reverse-phase LC-MS for all lipid extracts was performed using a Vydac C_{18} column (46 mm × 250 mm) and a LCQ Advantage ion-trap mass spectrometer (ThermoFinnigan), as described in ref. 2, except that the electrospray source operated at 1.8 kV and then at 210 C. All collision-induced dissociation MS experiments were performed on the ion-trap mass spectrometer as in ref. 2.

For supernatant lipids, mobile phase A was 20:80 methanol/acetonitrile with 1% acetic acid, mobile phase B was acetonitrile, and mobile phase C was 90:10 isopropanol/hexanes. All mobile phases contained 0.02% TFA, 0.1% formic acid, and 0.02% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIPA) (vol/vol). Beginning at a flow rate of 0.7 mL/min, a gradient was used beginning at 85% A, 15% B, and 0% C, adjusting linearly to 95% A, 15% B, and 0% C in 19 min, held at 95% A, 15% B, and 0% C for 2 min,

adjusting linearly to 1% A, 0% B, and 99% C in 8 min, held at 1% A, 0% B, and 99% C for 5 min, adjusting linearly to 15% A, 85% B, and 0% C in 2 min, adjusting linearly to 85% A, 15% B, and 0% C in 2 min, and held at 85% A, 15% B, and 0% C for 2 min. For analysis of the whole cell lipids, mobile phase A was 50:30:20 methanol/acetonitrile/water, and mobile phase B was 90:10 isopropanol/hexanes. Both mobile phases contained 0.02% TFA, 0.1% formic acid, and 0.02% HFIPA (vol/vol). A binary gradient was used beginning at a flow rate of 0.6 mL/min and 30% mobile phase B, held at 30% mobile phase B for 4 min, adjusting linearly over 21 min to 90% mobile phase B, adjusting linearly to 30% mobile phase B over 5 min, and held at 30% mobile phase B for 4 min, with the flow rate increasing linearly to 0.82 mL/min.

Macrophage and Animal Infections. Mouse macrophage J774 cells were infected with *M. bovis* BCG at a multiplicity of infection of 1:1 in 24-well culture dishes. After 4 h, cells were washed with PBS containing 1% BSA to remove extracellular bacteria. Cells were lysed by addition of PBS containing 1% Triton-20, and appropriate dilutions were plated on 7H10 agar containing hygromycin to estimate cfu per milliliter. For animal infections, 8- to 9-week-old female CB-17 SCID mice (The Jackson Laboratory) were injected i.v. with 10^5 of BCG-tet-*esx-3* mutant and 10^6 of wild-type *M. bovis* BCG (10:1 ratio). At 24 h or 30 days after infection, spleens and lungs were homogenized, and appropriate dilutions were plated on 7H10 agar. The BCG-tet-*esx-3* mutant bacteria were selected on 7H10 agar containing hygromycin, and total numbers of bacteria (wild-type + mutant) were estimated on 7H10 agar with no antibiotic.

Mycobactin Extraction from *M. smegmatis*. Solid medium for mycobactin extraction was made by boiling chelated Sauton's medium (without Tween-80) containing 0.1 μM $FeCl_3$ and 15 g/L agar. Saturated 7H9 medium cultures of *M. smegmatis* were diluted to OD_{600} of 0.15–0.2 in chelated Sauton's medium without Tween-80 containing 0.1 μM $FeCl_3$ and plated to form a bacterial lawn on solid Sauton's medium using 4-mm glass beads. After incubation at 37 °C for 4 days, the lawns were scraped into ethanol, and one drop of a saturated $FeCl_3$–ethanol solution was added. Mycobactin was extracted by adding two volumes of chloroform and washing with two volumes of water. To quantitate mycobactin, absorbance was measured at 450 nm. Total protein content of the samples was estimated by the Bradford assay and used to normalize the samples.

1. Ehrt S, et al. (2005) Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. *Nucleic Acids Res* 33:e21.

2. Matsunaga I, et al. (2004) *Mycobacterium tuberculosis pks12* produces a novel polyketide presented by CD1c to T cells. *J Exp Med* 200:1559–1569.

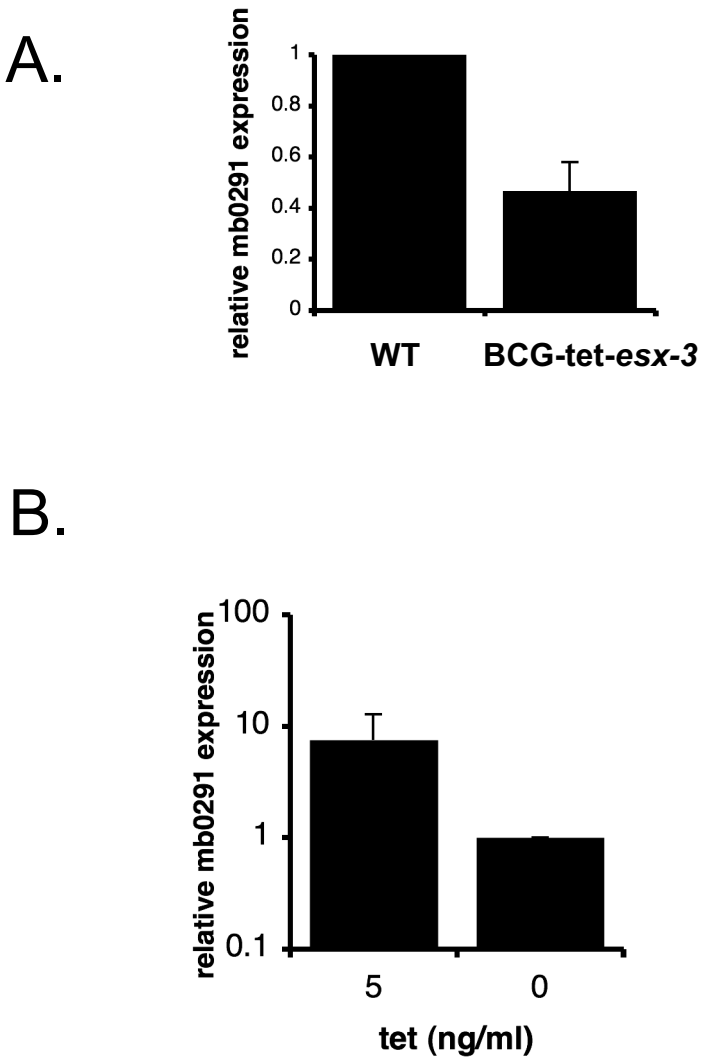


Fig. S1. Esx-3 expression in normal or low iron medium. (*A*) Quantitative RT-PCR analysis of *mb0291* expression of wild-type (WT) *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) and BCG-tet-*esx-3* mutant grown in 7H9 medium ($n = 3$). (*B*) Quantitative RT-PCR analysis of *mb0291* expression in BCG-tet-*esx-3* grown in low iron glycerol alanine salts medium in the presence or absence of the inducer anhydrotetracycline ($n = 3$). Data were normalized to *sigA* transcript levels. Error bars represent the standard deviations.

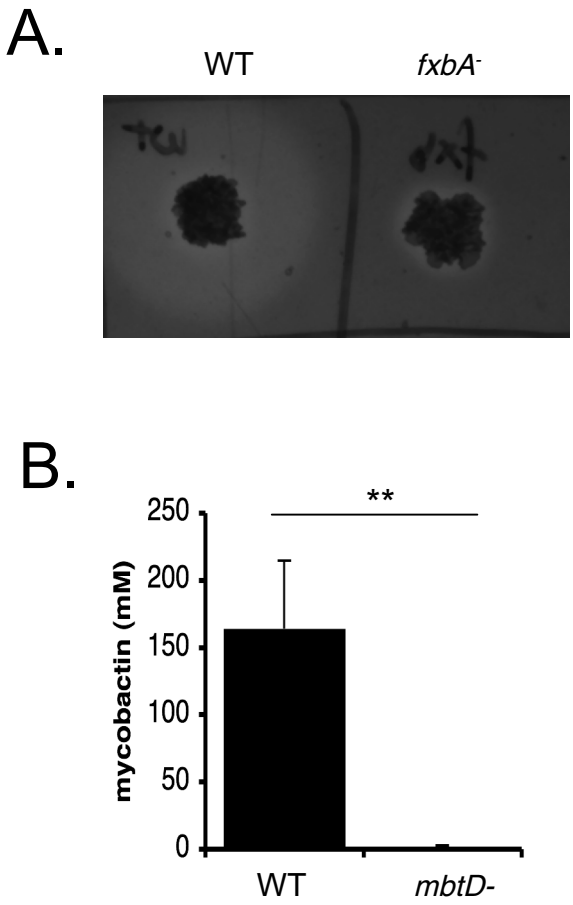
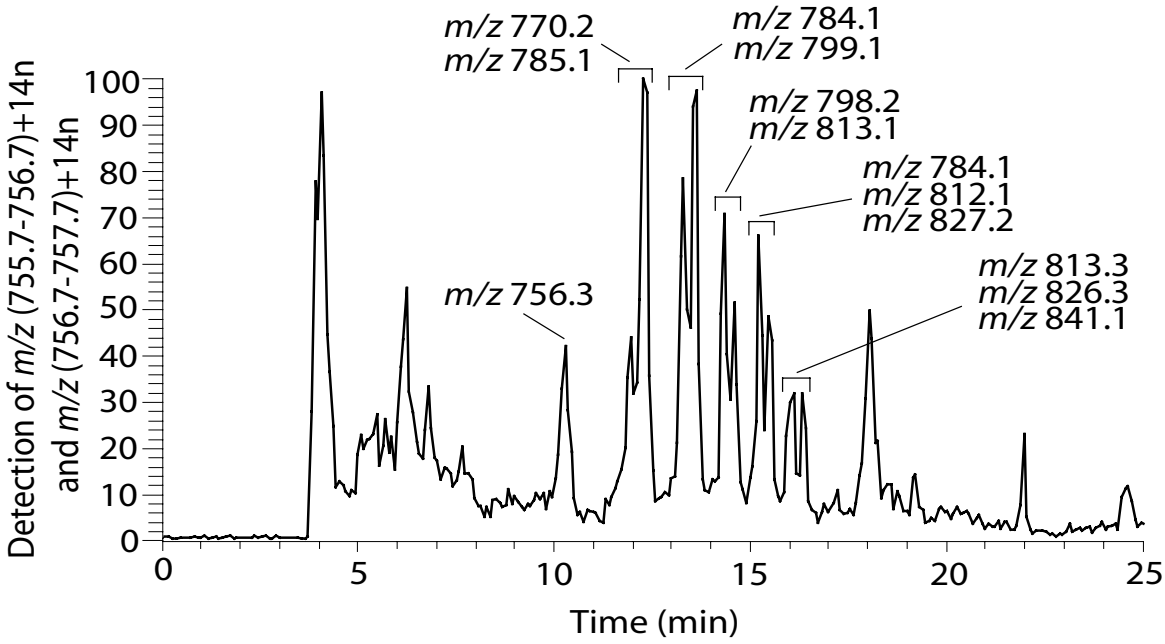


Fig. S2. *fxbA* and *mbtD* insertional mutants do not make exochelin and mycobactin, respectively. (*A*) Wild-type (WT) and *fxb*A::*pSES Mycobacterium smegmatis* were patched to Chrome Azurol S agar. Clearing around the patch indicates exochelin production. (*B*) Mycobactin abundance in WT and *mbtD*::*p402SK M. smegmatis* ($n = 3$; **, $P = 0.0296$).



Elution Time (min)	*m/z*	Adduct	Inferred Lipid Length	Composition
10.3	756.3	Na^+	$C_{8:0}$	$C_{35}H_{51}N_5O_{12}$
12.0	785.1	H^+	$C_{8:1}$	$C_{35}H_{47}N_5O_{12}Fe$
12.4	770.2	Na^+	$C_{9:0}$	$C_{36}H_{53}N_5O_{12}$
13.3	799.1	H^+	$C_{9:1}$	$C_{36}H_{48}N_5O_{12}Fe$
13.6	784.1	Na^+	$C_{10:0}$	$C_{37}H_{55}N_5O_{12}$
14.3	813.1	H^+	$C_{10:1}$	$C_{37}H_{50}N_5O_{12}Fe$
14.6	798.2	Na^+	$C_{11:0}$	$C_{38}H_{57}N_5O_{12}$
15.2	827.2	H^+	$C_{11:1}$	$C_{38}H_{52}N_5O_{12}Fe$
15.2	784.1	Na^+	$C_{9:0}$	$C_{38}H_{59}N_5O_{12}$
15.5	812.1	Na^+	$C_{12:0}$	$C_{39}H_{59}N_5O_{12}$
15.9	813.3	H^+	$C_{9:1}$	$C_{37}H_{50}N_5O_{12}Fe$
16.1	841.1	H^+	$C_{12:1}$	$C_{39}H_{54}N_5O_{12}Fe$
16.3	826.3	Na^+	$C_{13:0}$	$C_{40}H_{61}N_5O_{12}$

Fig. S3. Mass spectrometry of lipids extracted from *Mycobacterium smegmatis* supernatants. Initial MS of lipids extracted from conditioned culture supernatants showed two alkane series of carboxymycobactins that were either bound to iron and carrying an unsaturated dicarboxylic acid (*m/z* 756.2) or unbound to iron and carrying a saturated dicarboxylic acid (*m/z* 757.2). To simultaneously detect all members of these two alkane series, mass windows corresponding to the lowest molecular mass member of the series (*m/z* 755.7–756.7 and 756.7–757.7) and each of the four members of the series (n = 0, 1, 2, 3, 4) yield the ion chromatograms shown here and in Fig. 3*C*. Peaks corresponding to the elution of the various carboxymycobactin species are marked on the chromatogram, and further details about each molecule are given in the table. On the basis of collision-induced dissociation MS of individual ions, isobaric ions with differing elution times are isomers, and the earlier-eluting molecule has a larger lipid component, whereas the later-eluting molecule has a larger peptidic backbone. Comparison of the overall traces shown in Fig. 3 shows that the relative abundances of individual molecular species of carboxymycobactins to one another were unchanged after *esx-3* deletion.

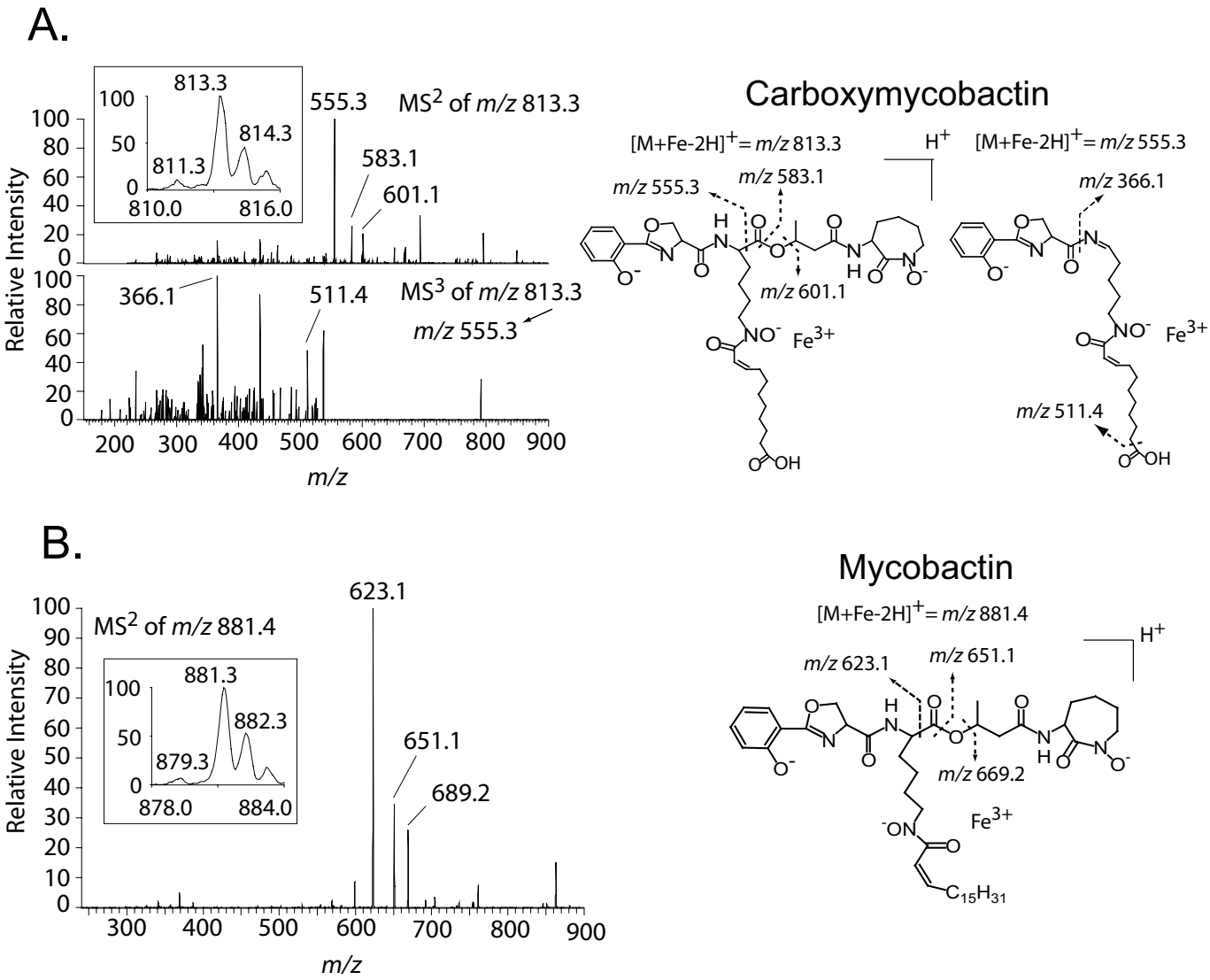


Fig. S4. Collision-induced dissociation (CID) MS analysis of carboxymycobactin and mycobactin. From among the lipids shown in Fig. 3*C*, one species of carboxymycobactin and one species of mycobactin were analyzed by CID-MS. Both spectra showed a detectable ion corresponding to 811.3 and 879.3, which correspond to the expected isotopes of siderophores bound to ^{54}Fe (*insets*). The MS^2 of both *m/z* 813.2 and *m/z* 881.5 generated fragments consistent with the predicted fragments derived from cleavage of the ester linkage of carboxymycobactin or mycobactin, respectively. In addition, MS^3 was performed on *m/z* 813.3, generating a fragment consistent with the loss of a carbonyl from its lipid chain, providing further evidence for its identity as carboxymycobactin. The fragmentation of both molecules was consistent with the fragmentation of authentic standards.

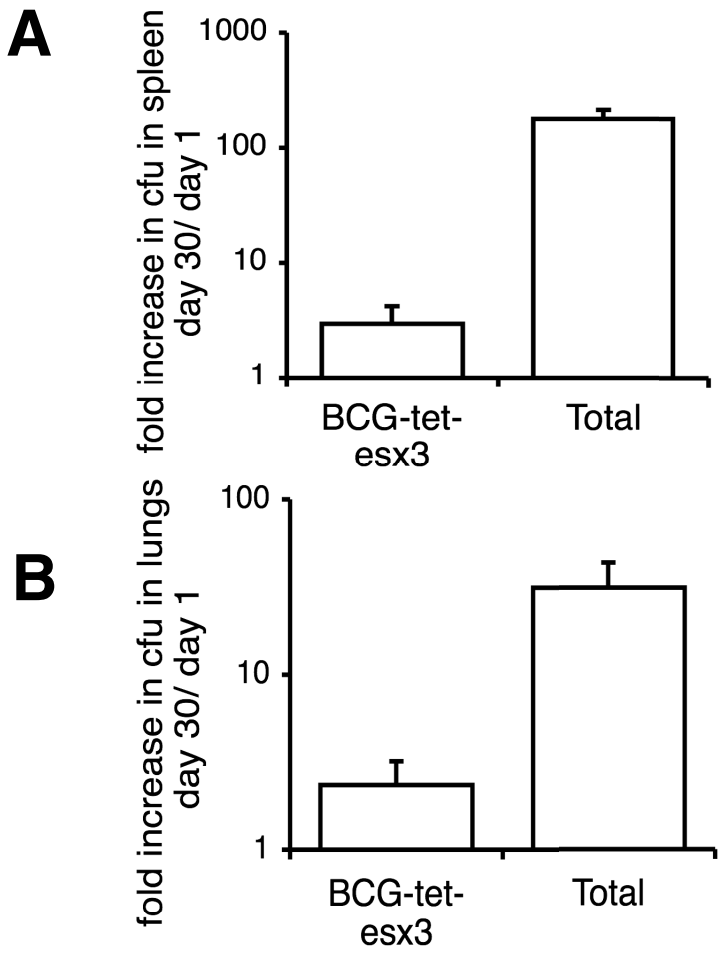


Fig. S5. Bacterial multiplication of wild-type *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) (Total = wild-type *M. bovis* BCG + BCG-tet-*esx-3*) and BCG-tet-*esx-3* in spleens ($n = 5$ per group) (*A*) and lungs ($n = 5$ per group) (*B*) of SCID mice after i.v. injection of a mixture of wild-type *M. bovis* BCG and BCG-tet-*esx-3* mutant at a ratio of 10:1. Error bars represent the standard deviations.

Table S1. Evidence that *esx-3* is essential in *Mycobacterium tuberculosis* and *Mycobacterium bovis* bacillus Calmette–Guérin (BCG)

	Hyg^R Suc^S colonies*	Confirmed single crosses†	Double crosses‡
M. tuberculosis			
esx-3	11	8	0
rv0283	30	0	0
M. bovis BCG			
esx-3	27	3	0
mb0291	24	0	0

We attempted to construct deletions of either *esx-3* or the first gene in the locus by cloning flanking regions from *M. bovis* BCG and *M. tuberculosis* into a suicide vector containing a hygromycin–chloramphenicol resistance cassette and the *sacB* counterselectable marker. Here, we tabulate the data for one representative experiment.

*The number of initial transformants that were resistant to hygromycin and sensitive to sucrose.

†The number of Hyg^R Suc^S colonies that were confirmed by PCR to contain the suicide vector in the correct genomic location. These colonies were then grown in medium containing no antibiotic and plated on sucrose.

‡The resulting Hyg^S Suc^R colonies were screened by PCR for the presence or absence of *esx-3*.

Table S2. Primers for quantitative RT-PCR

Gene	Primer sequences 5′-3′
Mycobacterium bovis bacillus Calmette–Guérin	
mb0291(rv0282)	F-atatggatccTGATGACGAACCAGCAGCACGA
	R-GGCAAGCACCGCGTTGCT
mbtB	F-CCCGGCTTATCGACACTTAC
	R-CCTGGACCTCGAAAATGGT
sigA	F-CCACGGGCGCCCAAGGATGC
	R-GAGTCGGCGGATGCGGTGAGTTCG
Mycobacterium smegmatis	
mbtB	F-CGATCAGCGCTTCCTGCTCAA
	R-AGATCACGCAGCACCGACAAA
mbtL	F-TGCAGTCAACATCCCCTGAT
	R-CAGCTCAGCAAATCCTCCTC
irtA	F-ACACGATGTCCGAGATGTCA
	R-GTCGTTCTCGTCGTGCTCTT
sigA	F-TACCTCAAGCAGATCGGCAAGGT
	R-TCGAGAACTTGTAGCCCTTGGTGT

F, forward; R, reverse.