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Mycobacterial Esx-3 is required for mycobactin-mediated iron acquisition

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The Esx secretion pathway is conserved across Gram-positive bacteria. Esx-1, the best-characterized system, is required for virulence of *Mycobacterium tuberculosis*, although its precise function during infection remains unclear. Esx-3, a paralogous system present in all mycobacterial species, is required for growth in vitro. Here, we demonstrate that mycobacteria lacking Esx-3 are defective in acquiring iron. To compete for the limited iron available in the host and the environment, these organisms use mycobactin, high-affinity iron-binding molecules. In the absence of Esx-3, mycobacteria synthesize mycobactin but are unable to use the bound iron and are impaired severely for growth during macrophage infection. Mycobacteria thus require a specialized secretion system for acquiring iron from siderophores.

secretion | siderophore

The thick, waxy mycobacterial cell wall functions as an exceptionally efficient barrier against a range of environmental and host insults. The relatively impermeable structure also poses a challenge, because it impedes the exchange of metabolites between the bacterium and its host during chronic intracellular infection. Mycobacteria have evolved both general and specialized systems to transport molecules across their cell walls (1). One such class of transporters is the Esx (type VII) secretion pathway (2).

The genome of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, contains five paralogous *esx* loci (3–5). Esx-1 is the best described. Substrates of this secretion system include the *esx-1*-encoded EsxB (Esat-6), EsxA (Cfp-10) and Rv3881c (6, 7), and EspA and EspB, encoded by an unlinked but evolutionarily related genetic locus (8, 9). Although Esx-1 and its substrates are required for virulence of pathogenic mycobacteria (10–12) and conjugation in *M. smegmatis* (13, 14), their precise contributions to these processes have not been determined (1, 2).

Even less is known about the other Esx systems. Preliminary characterization of Esx-5 has revealed that the system exports various proteins containing Pro-Pro-Glu (PPE) and polymorphic GC-rich sequences (PGRS) and modulates macrophage responses (15, 16). Neither protein export nor biological function has been reported for the products of *esx-2*, *esx-3*, and *esx-4*.

In a genome-wide scan of *M. tuberculosis* after growth on laboratory medium, we were able to recover transposon insertions in all of the *esx* loci except *esx-3* (17), suggesting that Esx-3 may be essential for mycobacterial growth in vitro. Previous reports suggest that expression of this locus is regulated by the metal-regulated repressors IdeR and Zur (18, 19). Besides divalent cation availability, in vitro growth and stress conditions such as acid, hydrogen peroxide, nitric oxide, SDS, stationary phase, and biofilms appear to alter *esx-3* expression (20–24).

We show here that Esx-3 is required for growth of *M. bovis* BCG and *M. smegmatis* under iron deprivation. Further, we demonstrate that Esx-3 is a secretion system and that export of *esx-3*-encoded substrates is iron-dependent. Genetic epistasis experiments reveal a link between iron acquisition via the

mycobactin siderophore pathway and Esx-3 protein export. Although mycobacteria that lack Esx-3 detect and initiate genetic programs in response to iron deprivation, they are unable to procure the metal from mycobactin or multiply normally in macrophages. Esx-3 is thus a specialized secretion system that is required for mycobactin-mediated iron acquisition and for survival during infection.

Results

Esx-3 Is Required for Growth under Iron-Limited Conditions. To investigate the apparent growth requirement for Esx-3 (25), we used gene set enrichment analysis (GSEA) (26) to test for coordinated transcriptional regulation of *M. tuberculosis* *esx* genes under 75 experimental conditions (27) that perturb growth in vitro. Significant up-regulation of *esx-3* gene expression occurs in response to five of the 75 treatments, four of which are iron chelators (Table 1). Furthermore, of all genomic windows for which we used GSEA to test transcriptional responses to chelation, *esx-3* is one of the most highly up-regulated, ranking between two loci that encode enzymes that synthesize the siderophore mycobactin (Table 2) (28, 29). These findings suggest that Esx-3 functions in response to iron deprivation in *M. tuberculosis*, and they correspond well with published data that show an increase in IdeR-regulated *esx-3* transcription in response to iron starvation (19).

To test whether Esx-3 is important for survival in iron-limiting conditions, we first sought to construct mutants defective in Esx-3 production. As predicted by the transposon insertion data (17), we were unable to recover *M. tuberculosis* or *M. bovis* BCG *esx-3* deletion mutants through homologous recombination, suggesting that this gene is indeed essential for growth (Table S1). Therefore, we generated a conditional *M. bovis* BCG *esx-3* mutant strain by replacing the native promoter upstream of the first gene in the locus, *mb0290*, with a tetracycline-regulated promoter (BCG-tet-*esx-3*; Fig. 1B). These mutants are able to grow without inducer (Fig. 2A), because the promoter is not repressed completely in the absence of tetracycline (Fig. S1A). We also found that, unlike pathogenic mycobacteria, the environmental organism *M. smegmatis* is able to tolerate deletion of the entire *esx-3* locus (Fig. 1C) and does not have a growth defect in complete medium. Development of these two experimental systems that bypass the growth requirement related to Esx-3 allowed us to probe Esx-3 function. Both mycobacterial strains

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The authors declare no conflict of interest.

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Table 1. Conditions affecting *esx-3* transcription

Treatment*	Transcription change direction	Nominal <i>P</i> value	False discovery rate <i>q</i> value†
111895	Up	0	0.00
Dipyridyl	Up	0	0.00
Deferoxamine	Up	0	0.04
Clofazimine	Up	0	0.04
Ascididemin	Up	0	0.03

Gene set enrichment analysis was performed as described in ref. 26. Expression changes with a nominal *P* value <0.05 and a false discovery rate *q* value <0.05 were considered significant.

*Treatments from ref. 27: 111895, dipyridyl, deferoxamine, and ascididemin are known chelators. Clofazimine is an antituberculosis drug with an unknown mechanism of action.

†False discovery rate *q* value is the fraction of observations expected to be false positives.

experience severe growth retardation in the absence of exogenous iron (Fig. 2 *A* and *B*).

Esx-3 Is an Iron-Responsive Secretion System. The organization of the mycobacterial *esx-3* and *esx-1* loci is similar: *esx-3* encodes EsxG and EsxH, which are paralogous to the small, secreted proteins EsxB (Cfp-10) and EsxA (Esat-6) encoded by *esx-1* (Fig. 1*A*) (5). Mutations in several *esx-1* genes abolish export of EsxB and EsxA (10, 30–35). To determine if the export of EsxH is dependent on Esx-3, we compared amounts of the heterologously-expressed, epitope-tagged protein in the cell-associated and supernatant fractions of wild-type and Δ *esx-3* *M. smegmatis*. We find that EsxH-c-myc secretion is dependent on the presence of Esx-3 (Fig. 2*D*). Protein export, moreover, is enhanced in both *M. smegmatis* and *M. bovis* BCG under low iron conditions (Fig. 2 *D* and *E*). Thus, Esx-3 does indeed function as a secretion system that functionally responds to iron deprivation.

Esx-3 Interacts with the Mycobactin Pathway of Iron Acquisition. Mycobacteria acquire iron from at least two classes of siderophore scavenging systems: exochelin and mycobactin (36). Although *M. smegmatis* produces both exochelin and mycobactin (37), pathogenic mycobacteria use only the pathway involving mycobactin and its structurally-related soluble form, carboxymycobactin (36). Expression of the mycobactin biosynthetic loci covaries with *esx-3* in our GSEA study and under several published conditions (20).

To test whether Esx-3 genetically interacts with either of the siderophore pathways, we constructed *M. smegmatis* mutants with insertions in *mbtD* (*mbtD*⁻), which encodes a polyketide

Table 2. Genomic windows up-regulated in low iron*

Gene range†	Nominal <i>P</i> value	False discovery rate <i>q</i> value‡	Annotation§
<i>rv1338-rv1362c</i>	0.00	0.03	<i>irtAB</i> , <i>fadD33</i> , <i>rv1347c</i>
<i>rv0278c-rv0302</i>	0.00	0.02	<i>esx-3</i>
<i>rv2380c-rv2403c</i>	0.00	0.05	Mycobactin synthesis
<i>rv2360c-rv2384</i>	0.00	0.05	Mycobactin synthesis
<i>rv1180-rv1204c</i>	0.00	0.05	Polyketide synthesis

*The 199 gene sets from overlapping 25-gene windows covering the *Mycobacterium tuberculosis* genome were tested by gene set enrichment analysis for transcriptional changes in response to chelator treatments from ref. 27.

†Identification of the first and last gene in the window are given. The gene sets consisted of these flanking genes plus annotated coding loci between them that have unique probes present on the arrays used in ref. 27.

‡False discovery rate *q* value is the fraction of observations expected to be false positives.

§Summary of the gene content of each window.

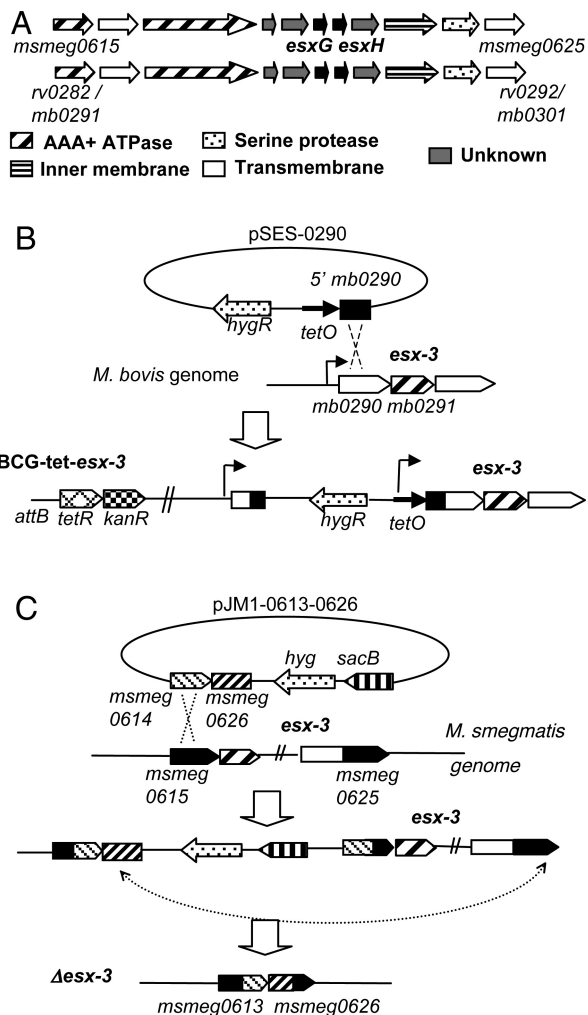


Fig. 1. Construction of the mycobacterial *esx-3* mutants. Schematic representation of the *esx-3* loci from *Mycobacterium smegmatis* (upper) and in *Mycobacterium tuberculosis*/*Mycobacterium bovis* bacillus Calmette–Guérin (BCG) (lower) (A) and strategies used to generate the *M. bovis* BCG *esx-3* conditional mutant (BCG-tet-*esx-3*) (B) and the *M. smegmatis* Δ *esx-3* mutant (C).

synthase required for mycobactin synthesis, or in *fxbA* (*fxbA*⁻), which encodes a formyl transferase required for exochelin synthesis (Fig. S2), and grew them without subculturing (in contrast to Fig. 2*B*) to produce an intermediate level of attenuation. Deletion of *esx-3* or inactivation of *mbtD* has little effect on *M. smegmatis* growth under these more permissive, low iron conditions (Fig. 3*A*). In contrast, inactivation of *fxbA* causes a marked growth defect, likely because exochelin is the primary iron acquisition system in *M. smegmatis* (37). Combining mutations in different iron acquisition pathways should produce a synthetic growth defect under conditions of iron starvation, whereas combining mutations in the same pathway should not. *fxbA*⁻ *mbtD*⁻, which lacks siderophores, and *fxbA*⁻ Δ *esx-3* both fail to grow in low iron medium (Fig. 3*A*). In contrast, the *mbtD*⁻ Δ *esx-3* strain does not exhibit an additive increase in low iron growth inhibition (Fig. 3*A*), strongly suggesting that Esx-3 functions in iron acquisition via the mycobactin pathway.

Requirement of Esx-3 for Iron-Limited Growth Can Be Complemented.

In BCG-tet-*esx-3*, the low iron growth defect is reversed by induction of Esx-3 (Fig. 2*A*). Because we were unable to complement *fxbA*⁻ Δ *esx-3* or Δ *esx-3* *M. smegmatis* with a cosmid containing the *M. tuberculosis* *esx-3* locus, we combined the

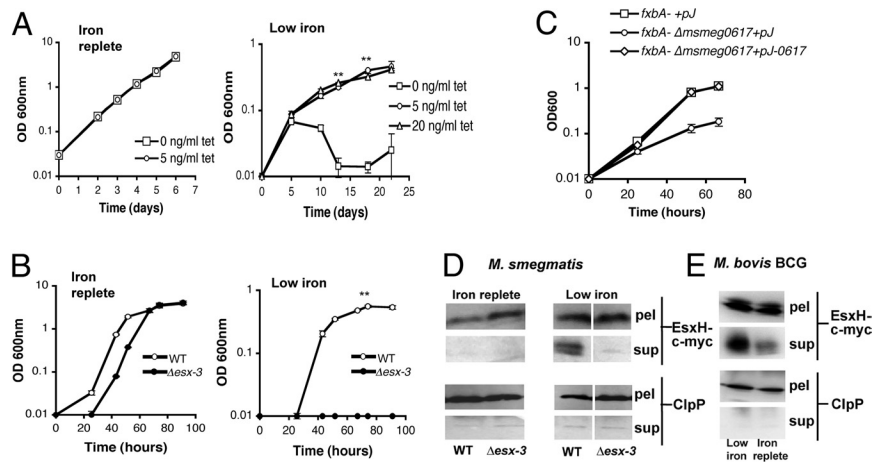


Fig. 2. *Esx-3* is required for *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) and *Mycobacterium smegmatis* growth and for secretion of *esx-3*-encoded proteins under iron-deprived conditions. Growth of BCG-tet-*esx-3* in iron-replete 7H9 medium or low iron glycerol alanine salts Tween-80 medium in the presence or absence of the inducer anhydrotetracycline ($n = 3$; **, $P = 0.0002$) (A). Growth of wild-type (WT) and $\Delta esx-3$ *M. smegmatis* in low iron (100 μ M 2,2'-dipyridyl) or iron-replete (12.5 μ M FeCl₃) Sauton's medium ($n = 3$; **, $P = 0.0014$) (B). Strains were subcultured in low iron Sauton's medium before inoculation. Growth of *fxbA*⁻ and *fxbA*⁻ $\Delta msmeg0617$ *M. smegmatis* containing pJEB402 with or without the *msmeg0617* gene in low iron Sauton's medium (C). Strains were not subcultured before inoculation. Anti-c-myc and anti-ClpP1P2 immunoblotting of cell lysates and culture supernatants of WT and $\Delta esx-3$ *M. smegmatis* containing pTetG-*esxGH-c-myc* (D) or WT *M. bovis* BCG containing pMV762-*esxG-6Xhis-esxH-c-myc* (E) in low iron or iron-replete Sauton's medium. All data are representative of at least three independent experiments. Error bars represent the standard deviations.

fxbA⁻ insertional mutation with a single gene deletion in the *esx-3* gene *msmeg0617*. The *M. tuberculosis* *esx-1* paralog *rv3870/3871* encodes an AAA+ ATPase that is required for Esx-1-mediated secretion, virulence, and conjugation in multiple my-

cobacterial species (1, 2). We find that *fxbA*⁻ $\Delta msmeg0617$ has a pronounced low iron growth defect that permits robust complementation by episomal expression of *msmeg0617* (Fig. 2C). Complementation of a single *esx-3* gene deletion further

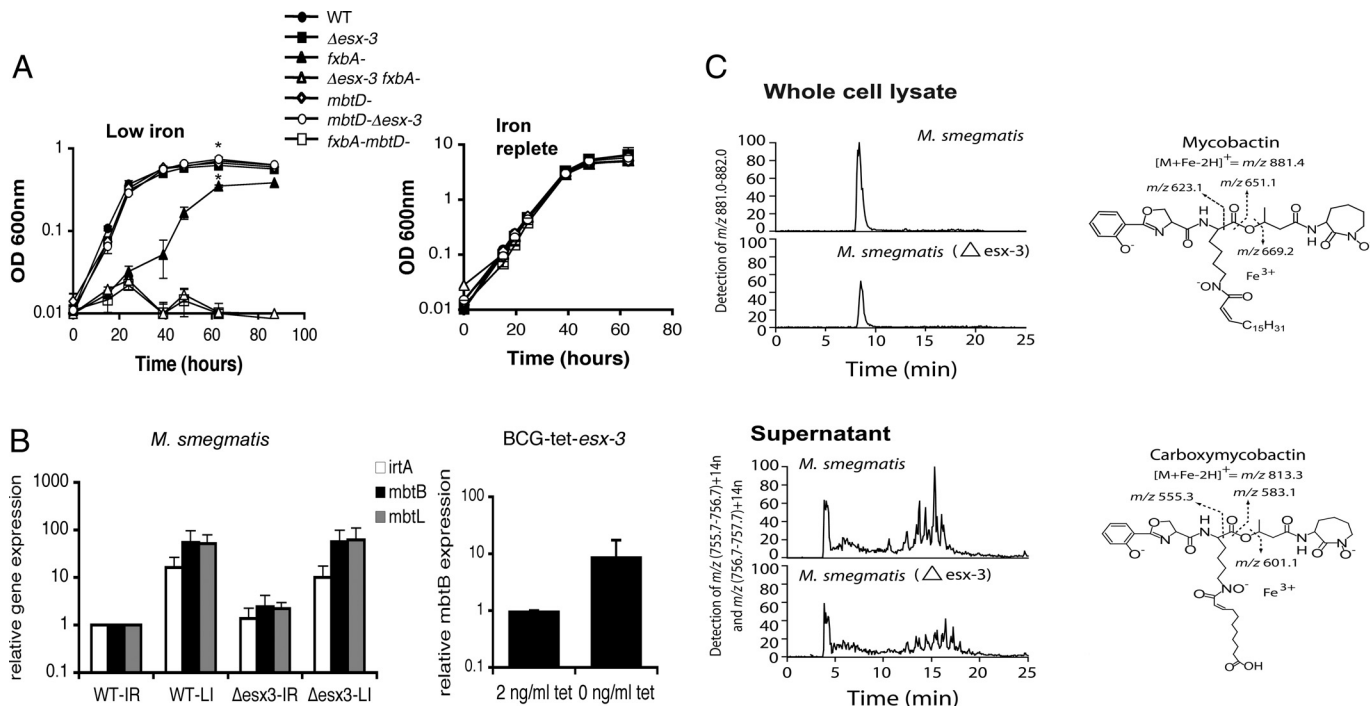


Fig. 3. *Esx-3* interacts with the mycobactin pathway but is not required for sensing iron or for siderophore synthesis. (A) Growth of wild-type (WT), $\Delta esx-3$, exochelin (*fxbA*⁻), and mycobactin (*mbtD*⁻) insertional biosynthesis mutants and double mutants (*fxbA*⁻ $\Delta esx-3$ and *fxbA*⁻ *mbtD*⁻) in low iron or iron-replete Sauton's medium ($n = 3$; *, $P < 0.001$). Strains were not subcultured before inoculation. (B) Quantitative RT-PCR for *mbtB*, *mbtL*, and *irtA* expression in WT and $\Delta esx-3$ *Mycobacterium smegmatis* ($n = 3$) in low iron (LI) or iron-replete (IR) medium and for *mbtB* in bacillus Calmette–Guérin (BCG)-tet-*esx-3* in low iron medium. Data representative of three independent experiments are shown. Error bars represent the standard deviations. (C) The LC-MS analysis of lipids extracted from the whole cell lysates of *M. smegmatis* with chloroform and methanol yielded mycobactin chromatograms in which the m/z (881.4), elution time, and fragments (right) correspond to the expected and actual measurements of an authentic standard. Conditioned supernatants were subjected to LC-MS detection in mass windows corresponding to the known masses of carboxymycobactins [m/z (756.7–757.7) + 14 n , where n is 0, 1, 2, 3, 4] and [m/z (757.7–758.7) + 14 n] (upper). The elution time and collision-induced dissociation MS fragmentation (right) of the putative carboxymycobactins are consistent with those of an authentic carboxymycobactin standard.

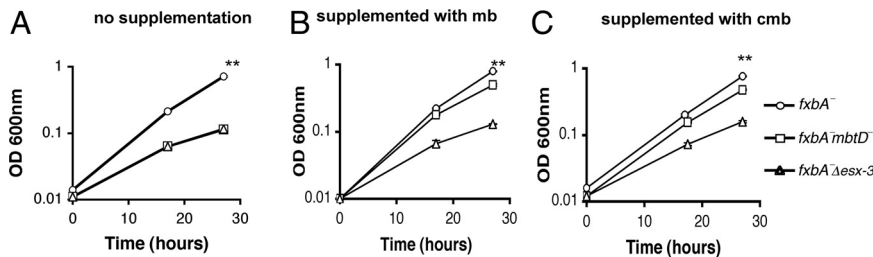


Fig. 4. *Esx-3* is required for optimal utilization of iron-bound mycobactin. Growth curves for *fxbA*⁻, *fxbA*⁻ *mbtD*⁻, and *fxbA*⁻ Δ*esx-3* *Mycobacterium smegmatis* in low iron Sauton's medium ($n = 3$; **, $P = 0.0005$) (A), in the presence of 10 ng/mL ferri-mycobactin S ($n = 3$; **, $P = 0.0017$) (B), or 20 ng/mL ferri-carboxymycobactin S ($n = 3$; **, $P = 0.0003$) (C). All data are representative of at least three independent experiments. Error bars represent the standard deviations.

supports a role for the secretion system in growth under iron-deprived conditions.

Impaired Iron Sensing or Reduced Mycobactin Abundance Do Not Account for the *esx-3* Defect. Several models could explain the genetic interaction between *Esx-3* and the mycobactin pathway. Mycobacteria that lack *Esx-3* might fail to increase mycobactin production in response to iron deprivation. A second possibility is that *esx-3* mutants are able to synthesize mycobactin but that the siderophore might not be exported from the cell or be functional. Finally, *Esx-3* might be required for utilization of iron-bound mycobactin.

Mycobacteria up-regulate the expression of siderophore biosynthesis genes when starved for iron (19). Could strains that lack *Esx-3* fail to sense low iron conditions and therefore fail to induce gene expression? To test this possibility, we compared transcript abundance of the iron-responsive genes *mbtB*, *mbtL*, and *irtA* in wild-type and Δ*esx-3* *M. smegmatis* in the presence or absence of iron. *mbtB* and *mbtL* reside in distinct genomic loci and encode mycobactin biosynthetic enzymes (28, 29), whereas *irtA* encodes part of an importer/exporter system for mycobactin (38, 39). Both strains increase transcription of these genes in response to iron deprivation (Fig. 3B). In fact, *mbtB* transcript abundance in uninduced BCG-tet-*esx-3*, which has low *esx-3* expression (Fig. S1B), is higher than that in the induced mutant (Fig. 3B). These data suggest that *Esx-3* is not necessary for iron sensing in *M. smegmatis* or *M. bovis* BCG but that its depletion in *M. bovis* BCG, which lacks alternate iron acquisition systems such as the exochelin pathway, may result in greater perceived iron starvation.

Although *esx-3* mutants do not appear to be defective for iron sensing, they might be unable to respond to iron deprivation by producing functional siderophores or, in the case of the soluble carboxymycobactin, by transporting them across the cell wall. To test whether *Esx-3* is required for mycobactin synthesis, we analyzed the siderophores in the whole cell lysates or conditioned supernatants of wild-type and Δ*esx-3* *M. smegmatis* with LC-MS. Analysis of samples that were normalized for mass and found to have unaltered signals for unrelated lipids showed that the ion current corresponding to molecules matching the expected mass of the iron-mycobactin complex (Fig. 3C) and related molecular variants are decreased moderately in the Δ*esx-3* strain. Carboxymycobactin detected as sodium adducts or as iron complexes in supernatants is detected at similar levels in wild-type and Δ*esx-3* *M. smegmatis* (Fig. 3C), and individual molecular variants in the spectrum of carboxymycobactins were preserved (Fig. S3). Collision-induced dissociation MS of the major mycobactin and carboxymycobactin species shows no changes in structure compared with those of standard compounds and prior analyses (Fig. S4). Although loss of *Esx-3* does not change the structures or alter the individual molecular species of mycobactin and carboxymycobactin, there is a small

but consistent decrease in the total pools of the siderophores. The moderate changes in total mycobactin abundance are unlikely to account for the striking low iron growth phenotype in the absence of *Esx-3*, because the *fxbA*⁻ *mbtD*⁻ *M. smegmatis* strain, which does not produce mycobactin, has a quantitatively similar growth defect in low iron to that of the *fxbA*⁻ Δ*esx-3* strain (Fig. 3A), which produces high amounts of mycobactin.

***Esx-3* Is Required for Mycobactin Utilization.** In response to iron deprivation, mycobacteria that lack *Esx-3* appear to produce mycobactin and carboxymycobactin and export carboxymycobactin appropriately. To test whether these mutants are impaired for utilization of the siderophore, we monitored growth of *fxbA*⁻ strains deficient in *Esx-3* or mycobactin production in the presence or absence of exogenous, iron-bound mycobactin S or carboxymycobactin S. Whereas the low iron growth of the *fxbA*⁻ *mbtD*⁻ strain can be rescued almost completely by purified mycobactin or carboxymycobactin, neither siderophore rescues the *fxbA*⁻ Δ*esx-3* strain to *fxbA*⁻ growth levels (Fig. 4A–C). Thus, *M. smegmatis* that lacks *Esx-3* is unable to use iron-bound mycobactin.

***Esx-3* Is Required for Growth in Macrophages.** Iron is limiting in many environmental conditions, particularly in an infected host (40). Consistent with a role for *Esx-3* in mycobactin-mediated iron acquisition, we find that BCG-tet-*esx-3* grows significantly more slowly in J774 macrophages in the absence of inducer (Fig. 5). Preliminary studies also suggest that BCG-tet-*esx-3* multiplies slower than wild-type *M. bovis* BCG in the spleen and lungs of SCID mice (Fig. S5A and B). Hence, *Esx-3* is likely important for *M. bovis* BCG growth both in vitro and in vivo.

Discussion

We demonstrate that *Esx-3*, a group of proteins of previously unknown function, is a specialized secretion system required for mycobactin-mediated iron acquisition. The wide variety of con-

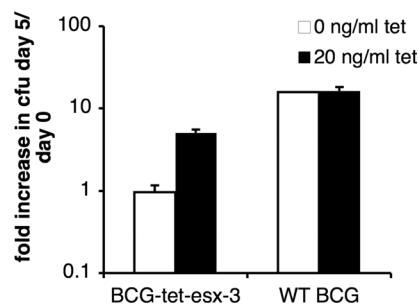


Fig. 5. *Esx-3* is required for mycobacterial infection of macrophages. Growth of wild-type (WT) *M. bovis* BCG and BCG-tet-*esx-3* in the presence or absence of anhydrotetracycline in J774 macrophages ($n = 3$).

ditions that perturb *esx-3* expression (18–24, 27) may, in part, reflect the ubiquitous need for iron in enzymes involved in multiple cellular processes (40). Alternatively, Esx-3 may transport additional substrates in addition to those involved in iron uptake.

The moderate reduction in total mycobactin in the absence of Esx-3 does not appear to be responsible for the growth defect seen under iron deprivation, given that the addition of purified mycobactin fails to rescue the *fxbA*⁻ Δ *esx-3* strain (Fig. 4). Further experimentation is necessary to determine whether the decrease in mycobactin pools in the absence of Esx-3 reflects mechanism or whether it is a consequence of perturbation of mycobacterial iron acquisition, normally a carefully orchestrated process.

How might a specialized secretion system like Esx-3 contribute to siderophore utilization? In other bacteria, siderophore-mediated iron acquisition requires the participation of multiple extracellular components (40). Although Gram-negative organisms have numerous mechanisms for siderophore secretion and uptake across the outer membrane, analogous systems for navigating the substantial cell wall of Gram-positive bacteria are poorly defined (40). The only known uptake system for mycobactin is via IrtB (38, 39), an ABC transporter confined to the cytoplasmic membrane (39). Esx-3 could have direct or indirect roles in iron transport. For example, the secretion system might export or position mycobactin-binding proteins in the cell wall. In this model, mycobacteria that lack Esx-3 or its transported substrates are defective for the delivery of iron-bound mycobactin to IrtB or possibly to alternate uptake systems. Alternatively, Esx-3 might regulate the activity of mycobactin transporters or have more global effects on the cell wall. The complex structure of the Gram-positive cell wall is a significant barrier to import or export. The Esx transport systems may provide a mechanism for moving compounds within and across the thick cell wall barriers of Gram-positive bacteria.

Materials and Methods

Bacterial Strains, Media, and Reagents. For iron starvation experiments, *M. bovis* BCG was cultured in iron-replete 7H9 medium (*SI Materials and Methods*) to late stationary phase, then diluted 1:100 or 1:250 in glycerol alanine salts Tween-80 medium lacking ferric ammonium citrate (41). Before infection experiments, bacteria were grown in 7H9 medium in the presence or absence of 5 ng/mL anhydrotetracycline. *M. smegmatis* was cultured in 7H9 medium, subcultured once in chelated Sauton's medium (*SI Materials and Methods*), and then diluted 1:1,000 in chelated Sauton's medium without antibiotics containing either 100 μ M 2,2'-dipyridyl or 12.5 μ M FeCl₃. For epistasis experiments, bacteria were diluted directly 1:1,000 from 7H9 medium into chelated Sauton's medium. The mycobactin and carboxymycobactin complementation experiments were performed similarly, except that 2,2'-dipyridyl was not added. Purified ferri-mycobactin S and ferri-carboxymycobactin S were kind gifts of Colin Ratledge (University of Hull, Hull, United Kingdom).

Mycobacterial Mutant Construction. Deletion and promoter replacements were constructed by transforming *M. tuberculosis*, *M. bovis* BCG, and *M. smegmatis* with suicide vectors containing antibiotic resistance markers and

the counterselectable marker *sacB*. Details of the selection process are contained in *SI Materials and Methods*.

Gene Set Enrichment Analysis. We used GSEA to test for coordinated transcriptional up- or down-regulation in 75 conditions in a published expression compendium (26, 27). Genes represented by identical or nearly identical microarray probes were removed from gene sets consisting of one *esx* locus each to avoid artifacts resulting from cross-hybridization. The GSEA enrichment was considered significant if it resulted in a nominal *P* value <0.05 and a false discovery rate <0.05. All five treatments meeting our criteria for significance resulted in increased transcription of *esx-3*. To assess the importance of the *esx-3* locus among all possible loci in *M. tuberculosis*, we performed GSEA using 199 gene sets, each containing 25 genes in overlapping sliding windows covering the entire *M. tuberculosis* genome. We do not believe that small differences in the relative rankings are meaningful at this level of resolution.

Immunoblotting. Wild-type and Δ *esx-3* *M. smegmatis* were transformed with pTetG-*esxGH-c-myc*, an episomal plasmid derived from pUV15tetOR (42), containing *esxG* and *c-myc* C-terminally tagged *esxH* from *M. smegmatis* under a tetracycline-inducible promoter. *M. bms* BCG were transformed with pMV762-*esxG-6XHis-esxH-c-myc*, an episomal plasmid that is derived from pMV762 (43) and contains poly-His N-terminally tagged *esxG* and *c-myc* C-terminally tagged *esxH* from *M. bovis* BCG. See *SI Materials and Methods* for details of protein isolation under low iron and iron-replete conditions and subsequent analysis.

RNA Extraction and Quantitative RT-PCR. Standard procedures are described in *SI Materials and Methods*.

Mass Spectrometry. *M. smegmatis* were diluted 1:500 from 7H9 medium into chelated Sauton's medium without Tween-80 containing 0.1 μ M FeCl₃. Cultures were grown to saturation, centrifuged, washed, and resuspended in the same medium. After 2 days of growth, bacteria were centrifuged to separate the whole cell and supernatant fractions, and the latter was filtered to remove bacteria and debris. Lipids were extracted from the culture supernatant by adding 6 N HCl and ethyl acetate and then centrifuging at 800 \times g for 30 min at room temperature. The upper organic phase containing the lipids was collected, evaporated to dryness, and resuspended in 1:1 chloroform/methanol. Lipids were extracted from the cell wall fraction as described in ref. 44. Details of the mass spectrometry are contained in *SI Materials and Methods*.

Macrophage and Animal Infections. Infection of mouse macrophage J774 cells by *M. bovis* BCG or BCG-tet-*esx-3* at a multiplicity of infection of 1:1 and mixed, i.v. infection of CB-17 SCID mice (The Jackson Laboratory) by 10⁵ cfu of BCG-tet-*esx-3* and 10⁶ cfu of wild-type *M. bovis* BCG (10:1 ratio) were assessed by standard procedures (*SI Materials and Methods*).

Statistical Analysis. Differences between groups were evaluated using a paired Student's *t* test.

Note Added in Proof. Recently, Serafini et al. (45) showed that Esx-3 is required for *M. tuberculosis* growth under iron-restricted conditions.

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