Disruption of Mycobactin Biosynthesis Leads to Attenuation of *Mycobacterium tuberculosis* for Growth and Virulence

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**Background.** Low iron availability in the host upregulates the *mbt* gene cluster of *Mycobacterium tuberculosis*, which is responsible for mycobactin biosynthesis. However, the biological significance of mycobactins in the growth of this pathogen and in disease progression has not been elucidated.

**Methods.** We have disrupted the *mbtE* gene (Rv2380c) in the *mbt* cluster to evaluate the importance of mycobactin biosynthesis in the growth and virulence of *M. tuberculosis*.

**Results.** The *mbtE* mutant (MtbΔmbtE) was unable to synthesize mycobactins, displayed an altered colony morphology, and was attenuated for growth in broth culture and in macrophages. Transmission electron microscopy revealed that MtbΔmbtE displayed an altered cell wall permeability. The growth characteristics and colony morphology of MtbΔmbtE were similar to wild type when the medium was supplemented with mycobactins or when MtbΔmbtE was genetically complemented with the *mbtE* gene. Moreover, guinea pigs infected with MtbΔmbtE exhibited a significantly reduced bacillary load and histopathological damage in the organs, in comparison to *M. tuberculosis*–infected animals.

**Conclusions.** This study highlights the importance of mycobactins in the growth and virulence of *M. tuberculosis* and establishes the enzymes of mycobactin biosynthesis as novel targets for the development of therapeutic interventions against tuberculosis.

**Keywords.** *Mycobacterium tuberculosis*; mycobactins; gene disruption; pathogenesis; drug targets.

Iron deficiency can prevent growth, and excess of iron can lead to the generation of reactive oxygen radicals. Hence, successful pathogens carefully control the levels of intracellular iron [1, 2]. *Mycobacterium tuberculosis* has developed an efficient mechanism to sequester iron from the host by secreting siderophores known as mycobactins. Mycobactins bind to iron more strongly than the iron-storage proteins of the host and play a crucial role of scavenging iron from the iron-limiting host environment [3, 4]. Although *M. tuberculosis* can uptake exogenous heme and use it as an iron source [5], mycobactin-mediated iron uptake remains its major iron-acquisition mechanism [6–10].

*M. tuberculosis* produces 2 classes of mycobactins: a less polar form of mycobactin (MBT), which remains associated with the cell wall, and a more polar form, carboxymycobactin (CMBT), which is secreted extracellularly [8]. Genome analysis of *M. tuberculosis* revealed the presence of a gene cluster known as the *mbt* cluster, that encompasses 10 genes, designated *mbtA* to *mbtJ*, that are exclusively responsible for mycobactin biosynthesis [4, 8, 11]. Several reports involving other organisms have shown that synthesis of siderophores is closely related to bacterial pathogenesis [12–15]. For *M. tuberculosis*, the *mbt* cluster is induced under low iron conditions, as well as in interferon γ (IFN-γ)–stimulated macrophages, thus indicating that *M. tuberculosis* can adapt its transcriptional machinery to environmental conditions by producing and secreting mycobactins required for increased uptake of iron by the pathogen [16].
and pathogenesis of M. tuberculosis has impaired replication in media containing low levels of iron and in macrophages [17]. In addition, the mbtE mutant of Mycobacterium smegmatis, a saprophytic mycobacterium, shows impaired growth in broth culture [18]. However, no studies have been performed to evaluate the importance of mycobactin biosynthesis during the survival of M. tuberculosis in the host.

In this study, we rendered M. tuberculosis incapable of synthesizing mycobactins by disrupting the mbtE gene (Rv2380c), which encodes a nonribosomal peptide synthetase. We demonstrate that mycobactin biosynthesis is essential for the survival and pathogenesis of M. tuberculosis.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

The bacterial strains and plasmids used in this study are listed in Table 1. Mycobacterial strains were grown on Middlebrook (MB) 7H11 agar supplemented with 10% OADC or in MB7H9 medium supplemented with 10% ADC (Difco Laboratories), 0.2% glycerol, and 0.05% Tween 80 at 37°C in an orbital shaker incubator at 200 rpm. An mbtE mutant of M. tuberculosis (MtbΔmbtE) was grown in the presence of 1 µg/mL of carboxymycobactin levels in the supernatant by using a universal chromosome assay and cloned at the Flp-1 Restriction site, respectively, flanking the hygromycin cassette in pYUB854 [20] to generate pVRΔM. A 3.4-kb (ΔmbtE::hyg) allelic exchange substrate was excised from pVRΔM by using SpeI and was electroporated into M. tuberculosis as described earlier [21] to generate the mbtE mutant of M. tuberculosis (MtbΔmbtE).

**Disruption of the mbtE Gene in M. tuberculosis**

Primers were designed to amplify (1) amplicon I, consisting of a 736-bp region encompassing the 3′ distal region of Rv2380c (254 bp) and sequence flanking Rv2380c downstream to its 3′ end (482 bp), and (2) amplicon II, consisting of a 688-bp region encompassing the 5′ proximal region of Rv2380c (221 bp) and sequence flanking Rv2380c upstream to its 5′ end (467 bp). Amplicons I and II were amplified by polymerase chain reaction and cloned at the KpnI/StuI and XhoI/SpeI restriction sites, respectively, flanking the hygromycin cassette in pYUB854 [20] to generate pVR1 (Table 1), a derivative of mycobacterial vector pSD5 [22], to generate pVR, prombtE (Supplementary Materials). MtbΔmbtE was transformed with pVR, prombtE to generate MtbΔmbtE Comp.

**Isolation of Mycobactins and Carboxymycobactins**

Mycobactins and carboxymycobactins were isolated from mycobacterial strains grown on minimal medium (MM) agar plates and in MM broth culture, respectively, as described previously [23, 24] (Supplementary Materials). In addition, mycobacterial strains were grown in MM broth and assayed for carboxymycobactin levels in the supernatant by using a universal chromo azurol S (CAS) plate assay and a liquid assay as described earlier [25–27].

<table>
<thead>
<tr>
<th>Strains and Plasmids</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>E. coli XL-1 Blue</td>
<td>endA1 gyrA96(nalR) thi-1 recA1 relA1 lacIqN44 F− [::Tn10 proAB + lacIq Δ(lacZI)M15] hsdR17(rK- mK-)</td>
<td>Stratagene (Heidelberg, Germany)</td>
</tr>
<tr>
<td>E. coli HB101</td>
<td>F− (gpt-proA) 62 leuB6 glnV44 ara-14 galK2 lacY1 (mcrC-mrr) rpsL20 (Strr) xyl-5 thi-1 recA13</td>
<td>Life Technologies (Carlsbad, CA)</td>
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<td>M. tuberculosis</td>
<td>Virulent strain of M. tuberculosis expressing recombineering proteins gp60 and gp61</td>
<td>[19]</td>
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<tr>
<td>MtbΔmbtE</td>
<td>M. tuberculosis mbtE mutant</td>
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<td>MtbΔmbtE Comp</td>
<td>MtbΔmbtE complemented with wild-type mbtE gene</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pYUB854</td>
<td>Cloning vector with hygromycin resistance gene cassette flanked with 2 multiple cloning sites</td>
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<td>pJV53</td>
<td>Mycobacteria–E. coli shuttle vector encoding recombineering proteins gp60 and gp61</td>
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<td>pVRΔM</td>
<td>pYUB854 with ΔmbtE::hyg</td>
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<td>pVR1</td>
<td>A derivative of pSD5 containing chloramphenicol resistance gene under mycobacterial trm promoter</td>
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<td>pVR, prombtE</td>
<td>pVR1 carrying mbtE gene expressed under native promoter</td>
<td>This study</td>
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Abbreviations: E. coli, Escherichia coli; M. tuberculosis, Mycobacterium tuberculosis.
**Electron Microscopy Studies**

For electron microscopy studies, samples were processed as described earlier [28], except that the samples were dehydrated in an ascending grade of acetone, infiltrated, and embedded in araldite CY 212. Sections were stained with alcoholic uranyl acetate and alkaline lead citrate and were observed under a Morgagni 268D transmission electron microscope at 80 kV.

**Infection of THP-1 cells**

Human monocytic THP-1 cells were cultured in complete Roswell Park Memorial Institute GlutaMAX medium (containing 10% heat inactivated fetal bovine serum and a 1% antibiotic-antimycotic mix; Gibco) and were differentiated into macrophages by exposure to 30 nM phorbol 12-myristate 13-acetate for 16 hours at 37°C in 5% CO₂. Activated macrophages were infected with *M. tuberculosis* and MtbΔmbtE separately, and the experiment was performed as described previously [19].

**Statistical Analysis**

For comparison between the groups, the nonparametric Kruskal-Wallis test, followed by the Mann-Whitney *U* test, 1-way analysis of variance with Tukey posttests, 2-way analysis of variance with Bonferroni posttests, and the Student *t* test were used, as appropriate. Differences were considered statistically significant when the *P* value was <.05. For statistical analysis and generation of graphs, GraphPad Prism 5 software was used.

**RESULTS**

**Disruption of mbtE Abrogates Mycobactin Biosynthesis in *M. tuberculosis***

To investigate the importance of mycobactin biosynthesis in *M. tuberculosis*, we disrupted the *mbtE* gene in the *mbt* cluster (Figure 1A and 1B). Verification of gene disruption was performed by PCR (Figure 1C), using genome-specific primers (Figure 1B). In addition, the PCR products (approximately 1.1 kb and approximately 1.0 kb) were sequenced by using the same sets of primers. The sequences of these amplification products confirmed the homologous recombination and disruption of the *mbtE* gene. Disruption of the *mbtE* gene was further confirmed by Southern blotting (Figure 1D), using the probe that recognized the region shown in Figure 1B. The mutant was designated MtbΔmbtE. MtbΔmbtE Comp strain was generated by transformation of MtbΔmbtE with mycobacterial plasmid expressing the *mbtE* gene (pVR.prombE) (Supplementary Materials and Supplementary Figure 1A and 1B).

To evaluate the effect of deletion of the *mbtE* gene, we first determined the production of siderophores by purifying the mycobactins and carboxymycobactins from *M. tuberculosis* and MtbΔmbtE grown on the deferrated MM agar plates and in MM broth culture.

The parental strain synthesized 1.53 mg of mycobactins per gram of wet cells, whereas no synthesis was observed in the case of MtbΔmbtE (Figure 1E). Our results are in agreement with earlier observations wherein *M. tuberculosis* synthesized a similar amount of mycobactins [30]. MtbΔmbtE Comp strain also synthesized 1.01 mg of mycobactins per gram of cells (Supplementary Figure 1E). These observations show that the inability of the MtbΔmbtE to synthesize mycobactins was due to the lack of the *mbtE* gene. In addition, we observed that the parental strain synthesized 6.53 µg of carboxymycobactins per milliliter of culture supernatant, whereas no carboxymycobactin was synthesized by the MtbΔmbtE strain (Figure 1F). Furthermore, the mycobactins synthesized by *M. tuberculosis* displayed an absorption spectra with absorption maximum of 450 nm, as reported earlier [24], whereas we could not detect any corresponding peak in the case of MtbΔmbtE (Supplementary Figure 2A). In addition, we performed a CAS liquid assay and a plate assay to detect carboxymycobactins secreted by *M. tuberculosis* and MtbΔmbtE. As expected, carboxymycobactins were produced by *M. tuberculosis* (27 carboxymycobactins units) when grown in minimal medium, whereas MtbΔmbtE produced no detectable levels of siderophores (Figure 1G and Supplementary Figure 2B and 2C). Thus, we demonstrate that *M. tuberculosis* synthesized both mycobactins and carboxymycobactins and that disruption of *mbtE* gene rendered *M. tuberculosis* incapable of synthesizing the mycobactins.

**Mycobactins Are Essential for Maintaining the Colony Morphology and Cell Wall Permeability of *M. tuberculosis***

Disruption of mycobactin biosynthesis resulted in alteration in the morphology of *M. tuberculosis* colonies on MB7H11 agar. MtbΔmbtE colonies were irregular and rugose and had elevated aerial growth in comparison to the colonies of the parental strain, which appeared relatively flat, rough, and granulated (Figure 2A). Moreover, MtbΔmbtE colonies appeared after a prolonged incubation period of 10 weeks. Supplementation of MB7H11 agar with Fe²⁺-CMBT restored the colony appearance and growth of MtbΔmbtE to extents similar to those of the parental strain (Figure 2A). Colonies of MtbΔmbtE Comp appeared on MB7H11 plates within 3–4 weeks and were similar to *M. tuberculosis* (Supplementary Figure 1C).
Since the disruption of mycobactin biosynthesis altered the colony morphology of *M. tuberculosis*, we further evaluated the cell wall structure of MtbΔmbtE mutant by transmission electron microscopy. MtbΔmbtE exhibited an altered cell wall permeability, with increased staining and no clearly visible demarcation of the cell wall. However, the parental strain displayed normal staining with a clearly visible boundary of the cell wall (Figure 2B). Supplementation of growth medium with Fe³⁺CMBT restored the cell wall permeability of the mutant to an extent similar to that of the parental strain (Figure 2B). The
changes observed in the colony morphology and cell wall permeability of Mtb\(\Delta\mbtE\) suggest that mycobactin biosynthesis plays an important role in maintaining normal cell wall architecture.

**Lack of Mycobactin Biosynthesis Results in Significantly Reduced Growth of \(M. \text{tuberculosis}\)**

To evaluate the importance of mycobactin biosynthesis in the growth of \(M. \text{tuberculosis}\), we compared the growth characteristics of Mtb\(\Delta\mbtE\) with that of the parental strain in broth culture. The parental strain grew logarithmically to an \(A_{600} \) of approximately 3.5, whereas the Mtb\(\Delta\mbtE\) mutant grew similar to the parental strain only to an \(A_{600} \) of 0.2–0.3, after which the absorbance declined (Figure 3A). To demonstrate that the growth defect of the mutant was attributable to its inability to synthesize mycobactins, we supplemented the medium with mycobactins. The growth of Mtb\(\Delta\mbtE\) was similar to that of the parental strain when medium was supplemented with either 1 \(\mug/mL\) of \(Fe^{3+}\)CMBT or 1 \(\mug/mL\) of \(Fe^{3+}\)MBT (Figure 3A and 3B). Genetic complementation of Mtb\(\Delta\mbtE\) with the \(mbtE\) gene restored its growth to an extent similar to that of the parental strain in MB7H9 medium (Supplementary Figure 1D). Growth of \(M. \text{tuberculosis}\) and Mtb\(\Delta\mbtE\) was also monitored in minimal medium with increasing concentrations of iron. Increasing the concentration of iron in the medium did not compensate for the absence of mycobactins and thus was unable to enhance the growth of Mtb\(\Delta\mbtE\) in broth culture. However, there was a substantial increase in the growth of \(mbtE\) mutant after increasing the concentration of iron in the presence of 1 \(\mug/mL\) of \(Fe^{3+}\)MBT (Figure 3C). Thus, our observations demonstrate that the severe growth defect of the \(mbtE\) mutant was solely attributable to the absence of mycobactin biosynthesis.

**Mtb\(\Delta\mbtE\) Exhibits a Severe Growth Defect in Human THP-1 Macrophages**

Human THP-1 macrophages were infected with the parental and mutant strains separately at a multiplicity of infection of 1:5. The Mtb\(\Delta\mbtE\) strain displayed a significantly reduced ability to infect macrophages, compared with the parental strain. Moreover, the mutant exhibited significant attenuation in its ability to survive and grow inside the macrophages (1.15 log\(_{10}\) CFU, 2.12 log\(_{10}\) CFU, 2.20 log\(_{10}\) CFU, 2.28 log\(_{10}\) CFU, and 2.31 log\(_{10}\) CFU on days 0, 2, 4, 6, and 8 after infection, respectively) as compared to the parental strain, which grew normally for 8 days (5.28 log\(_{10}\) CFU, 5.78 log\(_{10}\) CFU, 6.01 log\(_{10}\) CFU, 6.51 log\(_{10}\) CFU, and 6.53 log\(_{10}\) CFU on days 0, 2, 4, 6, and 8 after infection, respectively; Figure 4). These results

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**Figure 2.** Influence of mycobactin biosynthesis on the colony morphology and cell wall permeability of \(Mycobacterium \text{tuberculosis}\). **A**, Altered colony morphology of Mtb\(\Delta\mbtE\) in comparison to \(M. \text{tuberculosis}\). Mtb\(\Delta\mbtE\) displayed a distinct colony morphology in comparison to the parental strain. Supplementation of MB7H11 agar plates with 1 \(\mug/mL\) Fe\(^{3+}\)CMBT restored the colony morphology and growth of Mtb\(\Delta\mbtE\) to levels similar to those of the parental strain. The scale bars depict 2 mm. **B**, Altered cell wall permeability of Mtb\(\Delta\mbtE\), compared with \(M. \text{tuberculosis}\), as examined by transmission electron microscopy. Mtb\(\Delta\mbtE\) exhibited an enhanced cell wall permeability to the dye (alcoholic uranyl acetate and alkaline lead citrate), compared with the parental strain. Supplementation of growth medium with Fe\(^{3+}\)CMBT restored the cell wall permeability of the mutant to a level similar to that of the parental strain. The figure shows a representative transmission electron micrograph (original magnification, \(\times44\) 000).
demonstrate the importance of mycobactins in the intracellular survival of the pathogen within human THP-1 macrophages.

Disruption of Mycobactin Biosynthesis Attenuates Growth of \textit{M. tuberculosis} in Host Tissues

Guinea pigs were infected with \textit{Mtb}Δ\textit{mbtE} and the parental strain separately through the aerosol route. The animals were euthanized 4 weeks and 10 weeks after infection, and the influence of disruption of mycobactin biosynthesis on virulence was evaluated by bacterial enumeration in lungs and spleens.

Four weeks after infection, lungs and spleens of the guinea pigs infected with the parental strain exhibited a bacillary load of 4.68 log\textsubscript{10} CFU and 3.77 log\textsubscript{10} CFU, respectively, which increased to 4.87 log\textsubscript{10} CFU and 4.72 log\textsubscript{10} CFU, respectively, 10 weeks after infection (Figure 5A). However, no bacilli were recovered from the lungs and spleens of guinea pigs infected with \textit{Mtb}Δ\textit{mbtE} at both time points, even after incubation of the plates at 37°C for a prolonged period of 3 months.

Disruption of Mycobactin Biosynthesis in \textit{M. tuberculosis} Results in a Significantly Reduced Pathology

We assessed the influence of the disruption of mycobactin biosynthesis on the ability of \textit{M. tuberculosis} to cause disease and pathology. Four weeks after infection, the guinea pigs infected

\begin{figure}
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\caption{In vitro growth analysis of \textit{Mycobacterium tuberculosis} and \textit{Mtb}Δ\textit{mbtE} in broth culture. A, \textit{Mtb}Δ\textit{mbtE} is attenuated for growth in MB7H9 broth culture. \textit{M. tuberculosis} and \textit{Mtb}Δ\textit{mbtE} strains were inoculated separately in MB7H9 medium and incubated at 37°C for 16 days. The \textit{mbtE} mutant was also grown in the presence of 1 µg/mL of Fe\textsuperscript{3+}MBT. B, Growth of \textit{Mtb}Δ\textit{mbtE} in MB7H9 medium supplemented with mycobactins. The growth of \textit{Mtb}Δ\textit{mbtE} was compared with that of the parental strain by growing both in MB7H9 medium without and with 1 µg/mL Fe\textsuperscript{3+}MBT. C, Growth kinetics of \textit{M. tuberculosis} and \textit{Mtb}Δ\textit{mbtE} in minimal medium. \textit{M. tuberculosis} and \textit{Mtb}Δ\textit{mbtE} were grown in minimal medium with varying concentrations of iron (0, 50, 100, and 200 µM Fe\textsubscript{Cl}\textsubscript{3}6H\textsubscript{2}O). In addition, \textit{Mtb}Δ\textit{mbtE} was also grown in minimal medium with varying concentrations of iron but in the presence of 1 µg/mL of Fe\textsuperscript{3+}MBT. The growth of the strains was monitored by measuring the \textit{A}_{600 \text{nm}} \textit{nm} for 8 days. The experiment was repeated twice with 3 independent samples each time. The values are represented as the means ± standard error.}
\end{figure}
with the parental or the mutant strains exhibited comparable pathology in the liver and spleen, with the presence of numerous small tubercles, and moderate involvement in the lung, with occasional large tubercles (Figure 5B). Similarly, no histopathological differences were observed in lung and liver tissues of animals infected with the parental or MtbΔmbtE strains (Figure 6A).

Ten weeks after infection, a reduced gross pathology was observed in the organs of guinea pigs infected with MtbΔmbtE, compared with the organs of M. tuberculosis–infected animals. The lungs of M. tuberculosis–infected guinea pigs exhibited a heavy involvement, with numerous large tubercles with necrosis, whereas the lungs of animals infected with MtbΔmbtE exhibited a significantly reduced pathology, with minimal involvement. Moreover, the spleens of M. tuberculosis–infected guinea pigs exhibited substantial enlargement (splenomegaly), with the presence of numerous large and small tubercles. However, guinea pigs infected with MtbΔmbtE displayed a few extremely small (ie, pinhead-sized) lesions in the spleen, compared with those infected with the parental strain (P < .05; Figure 5B). The liver of M. tuberculosis– and MtbΔmbtE–infected guinea pigs displayed minimal involvement, with the presence of only a few visible tubercles.

Figure 5. Influence of disruption of the mbtE gene on the survival and pathogenesis of Mycobacterium tuberculosis in guinea pigs. A, Bacillary load in the organs of infected guinea pigs. The figure depicts the bacillary load in the lungs and spleens of guinea pigs infected with M. tuberculosis or MtbΔmbtE mutant 4 weeks (I) and 10 weeks (II) after infection. Each data point represents the log10 colony-forming units for an individual animal, and the bar depicts mean ± standard error. Data were analyzed by 2-way analysis of variance. *P < .05, **P < .01, ***P < .001.

B, Gross pathological lesions in the organs of infected guinea pigs. The figure depicts representative photographs of lungs, livers, and spleens from guinea pigs infected with M. tuberculosis or MtbΔmbtE euthanized 4 weeks (I) and 10 weeks (II) after infection. On the basis of the extent of disease involvement, size and number of tubercles, necrosis, and areas of inflammation, gross pathological scores were graded from 1–4 according to a modified Mitchison scoring system [29]. Each data point in the respective graph represents score of an individual animal, and the bar depicts median ± interquartile range for each group. The data were analyzed by the Mann–Whitney U test. *P < .05.
In agreement with the gross pathological observations, at 10 weeks after infection, animals infected with the parental strain exhibited enhanced histopathological damage as compared to animals infected with MtbΔmbtE. However, in the case of infection with MtbΔmbtE, lung parenchyma exhibited normal architecture, with infiltration of a few leukocytes (Figure 6B). Similarly, while the liver of M. tuberculosis–infected guinea pigs exhibited large areas of granulomatous inflammation, the animals infected with the mutant strain exhibited normal hepatic parenchyma (Figure 6B).

In addition, Ziehl-Neelsen staining of M. tuberculosis was performed in the lung tissues of guinea pigs 4 and 10 weeks after infection. Bacilli were detected in the Ziehl-Neelsen–stained lung tissues of guinea pigs infected with the parental strain. However, we could not detect any bacilli in the lung sections of animals infected with MtbΔmbtE (Figure 6C). These results demonstrate that the MtbΔmbtE mutant does not grow and sustain itself in the host tissues.

**DISCUSSION**

In this study, we disrupted the mbtE gene (Rv2380c) of M. tuberculosis, which encodes a nonribosomal peptide synthetase in the mbt cluster. Disruption of this gene renders M. tuberculosis incapable of synthesizing mycobactins. The MtbΔmbtE mutant displayed an altered colony morphology and was drastically affected in its ability to grow on agar medium and in broth culture, compared with the parental strain. Supplementation of
agar and broth medium with Fe³⁺CMBT or Fe³⁺MBT restored the growth of MtbΔmbtE to levels similar to that of the parental strain. Moreover, increasing the concentration of iron in the medium did not enhance the growth of the mutant, unless the medium was supplemented with mycobactins. Genetic complementation of MtbΔmbtE with the mbtE gene restored the in vitro growth phenotype of the mutant to levels similar to that of the parental strain. From these observations, it is evident that mycobactin-mediated iron acquisition is important for the normal growth of the pathogen. It is well documented that mutants of M. tuberculosis exhibiting altered colony morphology, which reflects subtle biochemical changes at the cell surface, can exhibit severe alteration in their virulence [31–33].

Transmission electron microscopy studies demonstrated that MtbΔmbtE displayed a much denser and darker staining of the cells along with the cytoplasm, emphasizing an altered cell wall permeability. Earlier, it was reported that mycobactins represent up to 10% of the cell mass and that 1% of these are present in the cell membrane itself [34]. Supplementation of growth medium with Fe³⁺CMBT restored the staining of MtbΔmbtE similar to that of the parental strain. The altered colony morphology, cell wall permeability, and growth characteristics of MtbΔmbtE suggest that in the absence of mycobactins, several iron-requiring systems of MtbΔmbtE might have been affected (emanating as a consequence of inability of the mutant to synthesize mycobactins). The restoration of normal growth, cell wall permeability, and colony morphology resulting from the addition of mycobactins in the medium suggest that, because of its essential role in procuring iron, mycobactin biosynthesis plays an important role in the biology of the pathogen. However, more work would be required to gather experimental proof to decipher whether the alteration of cell wall is a direct consequence of the absence of mycobactins or an indirect effect through its influence on a number of important enzyme systems that require iron.

The MtbΔmbtE mutant displayed a significantly reduced ability to infect and grow inside the human THP-1 macrophages in comparison to the parental strain, emphasizing that mycobactins are vital for mycobacterial growth. Gold et al have demonstrated upregulation of the mycobactin biosynthesis genes in macrophages [35]. It has also been reported that mycobactins can serve as extracellular siderophores within macrophages harboring mycobacteria, by diffusing through the membrane, scavenging iron from the intracellular pool, and delivering it to the phagosomes by lipid trafficking [7]. It can be hypothesized that MtbΔmbtE, because of disruption in mycobactin biosynthesis, is unable to acquire iron from the host, resulting in iron starvation. This severely affects its metabolism, resulting in a significant reduction in its ability to grow inside the macrophages. De voss et al have also reported that the mbtB mutant of M. tuberculosis was unable to synthesize mycobactin T and water-soluble mycobactin T. The mbtB mutant was impaired for growth in the medium containing a low level of iron but grew normally in the iron-replete medium. In addition, the mbtB mutant was also found to be impaired for growth in the macrophage-like THP-1 cells, suggesting that siderophore production may be required for the virulence of M. tuberculosis [17].

Our studies in guinea pigs provide further evidence that MtbΔambtE is highly attenuated in its growth and ability to cause pathology. The animals infected with the parental strain exhibited normal pathology, which increased from 4 weeks to 10 weeks after infection, as expected. However, in comparison, although the animals infected with MtbΔmbtE showed pathology 4 weeks after infection, the pathological damage was less 10 weeks after infection. In the case of infection with the parental strain, a substantial number of CFU was recovered from the lungs and spleen of animals at 4 and 10 weeks after infection, while no CFU was obtained from the animals infected with MtbΔmbtE at both time points. These observations demonstrate that the mutant strain could survive in the host only for a limited period. In addition, crucial proof of this came from the observation that, although Ziehl-Neelsen staining identified acid-fast bacilli in the lungs of animals 4 and 10 weeks after infection with the parental strain, no such identifiable bacilli were present in the lungs of animals infected with MtbΔmbtE. These observations demonstrate severe attenuation in the ability of the mutant to grow in the host and cause disease. Several studies have shown the relationship between iron supply and the growth of bacteria in animal models [36, 37]. Disruption of the iraAB locus, which is required for iron assimilation in Legionella pneumophila, yielded 1000-fold fewer bacilli in the lungs and spleens of guinea pigs infected with the mutant, compared with animals infected with the parental strain [38]. A null mutation in sitABCD locus involved in the synthesis of siderophores in Salmonella typhimurium has been shown to result in the attenuated phenotype in mice [39].

Our study demonstrates that disruption of mycobactin biosynthesis results in altered colony morphology, increased cell wall permeability, and a severe defect in the ability of M. tuberculosis to grow in broth culture and macrophages. In addition, studies in guinea pigs demonstrate that disruption of mycobactin biosynthesis renders the pathogen significantly attenuated for growth in the host, thus severely limiting its ability to cause disease, as supported by observations related to gross pathology and histopathological damage. Thus, this study highlights the importance of mycobactins in the normal physiology of M. tuberculosis in vitro and in the host and establishes the enzymes of mycobactin biosynthesis as novel targets for the development of therapeutic interventions against tuberculosis.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of...
data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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P. V. R., R. V. P., and A. K. T. conceived and designed the experiments. P. V. R., R. V. P., and P. C. performed macrophage experiments. R. K. and A. R. constructed pVR.prombtE. A. K. constructed the pVRAM plasmid. P. V. R. and R. V. P. conducted experiments involving guinea pigs. P. V. R. conducted all other experiments and analyzed the data. P. V. R. and A. K. T. wrote the manuscript. A. K. T. provided overall supervision throughout the study.

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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