 Participation of fad and mbt Genes in Synthesis of Mycobactin in Mycobacterium smegmatis

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Colonies of Mycobacterium smegmatis LR222 on iron-limiting (0.1 μM Fe) minimal medium agar fluoresce under UV light due to the accumulation in the cells of the deferri form of the siderophore mycobactin. Two mutants with little or no fluorescence, designated LUN8 and LUN9, were isolated by screening colonies of transposon (Tn611)-mutagenized M. smegmatis. Ferrimycobactin prepared from iron-restricted cells of the wild type had an Rf of 0.62 on high-performance thin-layer chromatography (HPTLC) and a characteristic visible absorption spectrum with a peak near 450 nm. Similar extracts from LUN8 cells contained a small amount of ferrimycobactin with an Rf of 0.58 on HPTLC and an absorption spectrum with the peak shifted to a wavelength lower than that of the wild-type ferrimycobactin. Nuclear magnetic resonance spectroscopy studies suggested that the LUN8 mycobactin may have an altered fatty acid side chain. Mutant strain LUN9 produced no detectable mycobactin. Neither mutant strain produced measurable amounts of excreted mycobactin, although both excreted exochelin (the mycobacterial peptido-hydroxamate siderophore), and both mutants were more sensitive than the wild-type strain to growth inhibition by the iron chelator ethylenediamine-di(o-hydroxyphenylacetic acid). The transposon insertion sites were identified, and sequence analyses of the cloned flanking chromosome regions showed that the mutated gene in LUN9 was an orthologue of the Mycobacterium tuberculosis mycobactin biosynthetic gene mbtE. The mutated gene in LUN8 had homology with M. tuberculosis fadD33 (Rv1345), a gene that may encode an acyl-coenzyme A synthase and which previously was not known to participate in synthesis of mycobactin.

The limited availability of iron in terrestrial and aquatic environments as well as in vertebrate hosts compels most pathogenic and nonpathogenic microorganisms to produce efficient iron acquisition systems, such as the uptake processes that are based on the iron-chelating agents called siderophores (4, 5, 10, 16, 32, 42, 47). With very few exceptions, the mycobacterial species (including both pathogenic and saprophytic forms like Mycobacterium tuberculosis and Mycobacterium smegmatis) synthesize siderophores of the mycobactin class (31, 44). The mycobactins are salicylic acid-derived siderophores containing a hydroxyarylloxazoline cap and two N-hydroxyamides that together form a high-affinity binding site for a ferric iron atom (44). A large number of mycobactins with different alkyl and acyl substituents at five positions on the core of the molecule have been described (44). The mycobactins can be divided into two subgroups based on lipid solubility. In the case of the lipophilic mycobactins (which are the wholly cell-associated form of mycobactin), the N-acyl moiety on the first hydroxylated lysine of the molecule is a long-chain fatty acid (C16 to C20) with a cis double bond (usually at position C-2), while in the more hydrophilic mycobactins (which are excreted from the cells), this acyl moiety is derived from a dicarboxylic acid that may be present as the free acid or the methyl ester (14, 15, 22, 23, 33, 50). The excreted, hydrophilic mycobactins have been called carboxymycobactins (22, 23), exomycobactins (29, 52), or oxochelins (14, 50), although Ralledge and Dover (32) have suggested that the exochelin designation be reserved for the excreted peptido-hydroxamate siderophores that are found only in saprophytic mycobacteria and which are structurally different from the mycobactins. This convention will be used here.

A cluster of 10 genes (designated mbtA-J) that encodes enzymes for the assembly of mycobactin was identified in the published M. tuberculosis genome sequence (6, 7, 30). Functions have been assigned to the mbt gene products that (together with phosphopantetheinyl transferases) account for the relevant biosynthetic steps to produce mycobactin, although the genes for synthesis of the variable aliphatic side chains and the required acyltransferase for attachment of these units have not been identified (8, 30). Five of the mbt genes form an assembly line of nonribosomal peptide synthetases (mbtB, mbtE, and mbtF) and polyketide synthases (mbtC and mbtD) that activate and elongate the monomers of the mycobactin core. An isochorismate synthase encoded by mbtI and an adenylylating enzyme, the MbtA protein, provide activated salicylate. Mycobactin chain initiation is thought to occur by acylation of an aryl carrier domain of the MbtB protein with activated salicylate (29, 30). By gene replacement, the mbtB gene was deleted and an antibiotic marker was inserted, yielding a mutant M. tuberculosis strain that failed to produce mycobactin and which was restricted for growth under low-iron conditions (11). These data link the mbt cluster to biosynthesis of mycobactin in M. tuberculosis, but functional characteriza-

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The analysis of the mycobactin biosynthetic genes did not reveal associated genes for iron or ferrimycobactin transport. Although there is ample evidence documenting mycobactin-mediated iron acquisition in mycobacteria (reviewed in references 10 and 32), the roles of the excreted (carboxymycobactin-exomycoactin) and the cell-associated form of mycobactin in iron transport are unclear. Uptake of iron from extracellular mycobactin appears to be by facilitated diffusion, and cell-associated mycobactin may serve as a short-term storage mechanism. Although there is ample evidence documenting mycobactin-mediated iron acquisition in mycobacteria, the exochelins may have an important iron uptake role in the terrestrial and aquatic environments preferred by the exochelin-producing saprophytic mycobacteria.

In this study, we report isolation and characterization of two M. smegmatis mutant strains generated by transposon insertion that are unable to produce (or have greatly diminished production of) mycobactin. The insertion site in one of the mutants (strain LUN9) was in the M. smegmatis orthologue of the mycobactin gene mbtE, while the insertion site for the other mutant (strain LUN8) was in the M. smegmatis orthologue of M. tuberculosis fadD33, a gene that heretofore had not been implicated in mycobactin synthesis. Both mutant strains still produce exochelin, but they were impaired for growth under iron-restricted conditions.

**MATERIALS AND METHODS**

**Bacteria, plasmids, media, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. E. coli cultures were routinely grown in Luria-Bertani broth or agar medium (27) at 37°C. M. smegmatis cultures were grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.); for plate (TSA) medium, agar (15 g per liter) was added. Antibiotics, when required, were added at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; hygromycin, 200 μg/ml for E. coli and 50 μg/ml for M. smegmatis.

For growth at defined iron concentrations, we used a minimal medium containing the remaining genes and their products has yet to be done (11).

Although both excreted and cell-associated mycobactins are produced by the saprophyte M. smegmatis (33), the predominant extracellular siderophore of this organism is exochelin MS, an ornithine-derived trihydroxamate (41). A similar peptido-hydroxamate molecule, exochelin MN, is produced by Mycobacterium neoaurum (40). Genes for biosynthesis, excretion, and uptake of exochelin have been cloned from M. smegmatis and sequenced (13, 51, 52). The genes, named fxbA, fxbB, and fxbC, were proposed to encode the cytoplasmic membrane permease for the transport of ferriexochelin into M. smegmatis (13). Several genes have been shown to be involved in exochelin biosynthesis and export. One (fxbA) was suggested to encode an enzyme that adds a formyl group in the synthesis of exochelin. The other (fxbB) is 3', and possibly -D) encode non-ribosomal peptide synthetases that likely form a complex with an ABC transporter known as ExiT (or ORF1 and -2) to synthesize and export the peptide siderophore. The exochelins have not been found in pathogenic mycobacteria (32), and the need for both exochelin and mycobactin in saprophytic mycobacteria is unclear. The exochelins may have an important iron acquisition role in the terrestrial and aquatic environments preferred by the exochelin-producing saprophytic mycobacteria.

The insertion site in one of the mutants (strain LUN9) was in the M. smegmatis orthologue of the mycobactin gene mbtE, while the insertion site for the other mutant (strain LUN8) was in the M. smegmatis orthologue of M. tuberculosis fadD33, a gene that heretofore had not been implicated in mycobactin synthesis. Both mutant strains still produce exochelin, but they were impaired for growth under iron-restricted conditions.
taining (per liter): glucose, 10 g; (NH₄)₂SO₄, 1 g; KH₂PO₄, 3.5 g; KH₂PO₄, 1.5 g. To lower the trace metal contamination, the medium was treated with Chelex-
100 (Bio-Rad Laboratories, Hercules, Calif.) by previous reported methods (1).
Before use, the medium was supplemented with 0.1% sterilized Tween 80 and with
filter-sterilized high-purity salts (Johnson-Matthey, Inc.), of magnesium (830 μM), manganese (40 μM), zinc (0.3 μM), and iron (various
concentrations). The glassware was soaked with 0.025 M tetradsodium EDTA
overnight to remove contaminated metals and rinsed extensively with laborato-
ry-grade water.

Molecular biology techniques. Standard molecular biology techniques and procedures were followed as described by Sambrook et al. (37) and Davis et al. (19).
Enzymes were used as directed by the manufacturers with the buffers pro-
vided. Plasmid DNA from E. coli cells was prepared by the alkaline lysis method
(3) and was purified by RNase treatment and phenol extraction. Isolation of
plasmid DNA from M. smegmatis (21) was essentially like that from E. coli, i.e.,
alkaline lysis, except that the cells were first disrupted (beaten for 2 min) by glass
beads in a Mini-BeadBeater (Biospec Products, Bartlesville, Okla.).

Introduction of plasmids into M. smegmatis was by electroporation (21). Bac-
teria suspended in 10% glycerol electroporation buffer with 1 μg of DNA were
subjected to a single pulse using the Bio-Rad Gene Pulser (Bio-Rad Laborato-
ries) set at 2.5 kV, 25 μF, with the pulse controller resistance set at infinity. The
content of the cuvette was diluted into 5 ml of TSB and incubated at 42°C. After
incubation for 3 days, aliquots were plated on antibiotic-TSA plates; transfor-
mants appeared after incubation for 3 days.

Genomic DNA from M. smegmatis cells, disrupted with a Mini-BeadBeater
cell disruptor, was isolated with an Easy-DNA kit (Invitrogen Life Technologies,
Carlsbad, Calif.) and then was purified by RNase treatment and phenol extrac-
tion. Alternatively, genomic DNA was prepared as described elsewhere (4).
Cells were lysed with sodium dodecyl sulfate, and proteins were removed by
digestion with proteinase K. Cell wall debris and remaining proteins were re-
moved by precipitation with cetyltrimethylammonium bromide, and high-molec-
ular-weight DNA was recovered by isopropanol precipitation. M. tuberculosis
DNA was obtained from John T. Belisle and was produced through funds from
the National Institute of Allergy and Infectious Diseases, National Institutes of
Health, contract 1-AI-25147 entitled “Tuberculosis Research Materials.”

Southern hybridization probes used in this study were prepared by digesting
plasmids carrying the desired gene with the appropriate restriction enzymes,
separating the DNA fragments by agarose gel electrophoresis, excising the DNA
probe band, and purifying it by phenol extraction. The DNA probe was radio-
abeled with [32P]dCTP by the random primer method using the
and hybridization were performed according to the instructions of the manufac-
turer of the transfer membrane (NEN Life Science Products, Boston, Mass.).

Mutagenesis and screening for mycobactin mutants. Transposon mutagenesis of M. smegmatis LR222 was performed as described by Guilhot et al. (17).
Plasmid pC79 containing Tn611 was introduced into M. smegmatis by electro-
poration. Transformants were selected at 37°C on TSA plates plated on antibiotic-
TSA plates; transformants were purified by phenol extraction. The DNA probe was radio-
abeled with [32P]dCTP by the random primer method using the
and hybridization were performed according to the instructions of the manufac-
turer of the transfer membrane (NEN Life Science Products, Boston, Mass.).

Nucleotide sequence accession numbers. The nucleotide sequences of the M. smegmatis LR222 mbdE genes and the acp/fadE gene cluster were
obtained from GenBank database accession numbers AY225312 and AF277395, respectively.

RESULTS
Isolation of mycobactin mutants. M. smegmatis LR222, a strain derived from M. smegmatis mc²6 (2), was mutagenized with Tn611, and the insertional library was screened for loss of mycobactin production on low-
iron (0.1 μM) minimal medium containing glutamate, glycerol, and kanamycin. After 5
The capacities of the mycobactin mutant strains LUN8 and LUN9 to overcome iron restriction imposed by the iron-chelating agent EDDA were determined and compared to those of wild-type M. smegmatis strain LR222 contained an approximately 17-carbon, 2,3-unsaturated aliphatic side chain at the R1 position. Signals from the 2,3-hydrogens at the double bond in the R1 side chain of the wild-type siderophore were seen as a set of correlated signals at δ 6.0 and 6.3 ppm (downfield from standard tetramethylsilane) in one- and two-dimensional proton NMR spectra. These signatures were not seen in the mycobactin produced by the LUN8 strain. Therefore, a saturated alkyl side chain, probably of wild-type carbon length, was predicted to be attached at the R1 position of the mycobactin made by strain LUN8. The resulting alkane group at this position could alter solubility, resulting in the slightly lower \( R_f \) value observed for LUN8 mycobactin on HPTLC plates (Fig. 1). The complete structural description of the mycobactin produced by strain LUN8 is in progress and will be the subject of another publication.

To assess production of the excreted form of mycobactin (exomycobactin or carboxymycobactin) by the mutant strains LUN8 and LUN9, chloroform extracts of the cell-free filtrates of low-iron cultures were examined by HPTLC. Excreted mycobactin with a characteristic \( R_f \) value of 0.23 was detected in culture filtrates of the wild-type strain LR222 and the previously described exochelin mutants (52) LUN1 and LUN2. Exomycobactin was not detectable in culture filtrates of either strain LUN8 or LUN9, although strain LUN8 may have excreted exomycobactin in amounts below the detection threshold of the assay. Both strains LUN8 and LUN9 excreted the peptido-hydroxamate siderophore exochelin, which was detected by assay of the cell-free filtrates of low-iron cultures with the liquid chrome azurol S shuttle assay of Schwyn and Neilands (39).

**Sensitivity of mycobactin mutant strains to iron restriction.**

The capacities of the mycobactin mutant strains LUN8 and LUN9 to overcome iron restriction imposed by the iron-chelating agent EDDA were determined and compared to those of wild-type M. smegmatis strain LR222, by the previously described (52) mutant strains LUN1 and LUN2 that are unable to produce the siderophore exochelin, and by strains LUN8 and LUN9. Mycobactin \( J \) was included as a reference.

Lack of mycobactin production by strain LUN9 and production of an altered mycobactin by strain LUN8. To assess production of cell-associated mycobactin by strains LUN8 and LUN9, ethanolic extracts of cells grown in low-iron (0.1 \( \mu \)M) minimal medium were analyzed by silica gel HPTLC. Strain LUN9 produced no detectable mycobactin, whereas strain LUN8 produced a ferric-chelating molecule that migrated more slowly (\( R_f \), 0.58) than mycobactin produced by the parental, wild-type strain LR222 (\( R_f \), 0.62) (Fig. 1). When the mycobactin preparations from strains LUN8 and LR222 were mixed and chromatographed, separate bands were noted, confirming that the mycobactin-like product from strain LUN8 was distinct from mycobactin produced by the wild type (data not shown). The previously described (52) mutant strains LUN1 and LUN2 that fail to make the peptido-hydroxamate siderophore exochelin also were analyzed and found to produce mycobactin with a wild-type \( R_f \) (Fig. 1).

The ferric-chelating material prepared from strain LUN8 was concentrated and purified by adsorption to, and elution from, alumina. Mycobactin from the wild-type strain LR222 was likewise purified. The electronic absorption spectrum of ferrimycobactin from LR222 showed a broad peak with maximum absorbance near 450 nm, characteristic of the ferrimycobactins (Fig. 2). The ferric chelate of the substance produced by strain LUN8 also displayed a broad peak, with the maximum shifted to a wavelength somewhat lower than that of the wild-type ferrimycobactin (Fig. 2). Comparison of the absorption maxima of the identically prepared samples from both strains indicated that strain LUN8 made a mycobactin-like material at about 1/10 the production level of wild-type mycobactin. Preliminary NMR spectroscopy of deferrated mycobactin isolated from both the wild-type and the LUN8 strains depicted molecules with the typical ortho-salicylate aromatic moiety, similar to mycobactin \( S \) previously described by Ratledge and Dover (32).

**FIG. 1.** HPTLC of cell-associated ferrimycobactins produced by wild-type *M. smegmatis* strain LR222, by the previously described (52) mutant strains LUN1 and LUN2 that are unable to produce the siderophore exochelin, and by strains LUN8 and LUN9. Mycobactin \( J \) was included as a reference.

**FIG. 2.** Absorption spectra of identically prepared samples of ferrimycobactin-like material produced by *M. smegmatis* mutant strain LUN8 (A) and of ferrimycobactin prepared from wild-type *M. smegmatis* strain LR222 (B). Wild-type ferrimycobactin was diluted 10-fold for this scan.
of the previously described exochelin mutants LUN1 and LUN2 (52). The MIC of EDDA for the wild-type strain LR222 was more than 250 μg of EDDA/ml, whereas the MICs for both strains LUN8 and LUN9 were lowered to approximately 50 μg of EDDA/ml. On the other hand, the MICs for the exochelin mutants LUN1 and LUN2 were essentially identical to that for the wild type, at 250 μg of EDDA/ml. Control studies showed that EDDA inhibition of the M. smegmatis strains was reversed by iron salts, confirming that this inhibition was due to iron restriction. These data suggest that mycobactin synthesis but not exochelin production was necessary to overcome iron limitation caused by EDDA.

**Cloning and identification of mycobactin genes.** Insertional mutagenesis with Tn611 was confirmed by the previously described strategy (17, 52) of digesting the genomic DNAs from the mutants with PstI and performing Southern hybridizations using the insertion sequence IS6100 as a probe (Fig. 3). Colony blotting was used to clone the transposon-flanking regions of the insertion mutants. Both DNA fragments from the PstI digestion were too short to give enough information for further work. Therefore, we used the enzymes SmaI and SacI to cut the genomic DNA and performed Southern blotting using IS6100 as a probe. The results showed that both mutants had different hybridization patterns from the plasmid control (Fig. 3). By using the same probe, a 3.5-kb SacI fragment (pWEN3) was cloned from LUN8 and a 4.4-kb SmaI fragment (pARC6) was cloned from LUN9. Other fragments seen in the blot were vector sequence. A portion of the LUN8 genome, downstream from the insertion, was cloned by taking advantage of the plasmid vector that was integrated into the chromosome along with Tn611. This integrated vector carries an E. coli origin of replication and resistance genes for kanamycin and streptomycin. By cutting LUN8 DNA with EcoRI, ligating, transforming E. coli cells, and selecting for kanamycin resistance, a plasmid with Tn611 was recovered. This plasmid carries the genomic region on the other side of the LUN8 Tn611 insertion from that DNA that yielded pWEN3.

The cloned insertion flanking regions from both LUN8 and LUN9 were sequenced. Nucleotide sequencing showed that the insertion flanking region from LUN8 shared homology with the mycobactin biosynthesis genes mbtD and mbtE of M. tuberculosis (Fig. 4). The mutated gene from LUN8 encoded the N-terminal portion of a protein with homology to M. tuberculosis FadD33, an acyl-coenzyme A (CoA) synthase (Fig. 5). This DNA also encoded an acyl carrier protein (M. tuberculosis Rv1344 orthologue) of unknown function. In addition to the 3' end of fadD33, the DNA cloned into pML222 encoded an orthologue of the M. tuberculosis FadE14 protein.

**Complementation of LUN8.** Plasmid pBLT1344 carrying PCR-amplified acp/fadD/fadE DNA from the wild-type M. smegmatis strain LR222 and subclones of pBLT1344 were electroporated into strain LUN8, and the resulting transformants were observed for fluorecence under UV (245 nm), indicative of mycobactin production (Fig. 6). Extraction and HPTLC analysis of ferrimycobactin produced by a fluorescing LUN8/ pBLT1344 transformant confirmed its production of wild-type levels of mycobactin with a wild-type R. Complementation of strain LUN8 by the subclones showed that only those constructs that encoded both of the complete fadD and fadE genes restored mycobactin synthesis in strain LUN8 (Fig. 6). Transformation of strain LUN8 cells with the PCR-amplified M. tuberculosis acp/fadD/fadE orthologues carried on plasmid pMLNV1344 failed to yield fluorescing colonies, indicating that the M. tuberculosis genes were unable to complement the M. smegmatis strain.

**In silico analysis of mycobactin gene clusters.** Analysis of DNA sequences indicated that the flanking regions from LUN9 had homology with mycobactin genes mbtD and mbtE from M. tuberculosis (Fig. 4). MbTD belongs to the polyketide synthase superfamily of proteins that contain consensus sites for posttranslational phosphopantetheinylation that are involved in acyl group transfer (10, 30). MbtE shares homology with nonribosomal peptide synthetases and has a single amino-acid adenylation domain that activates the lysine residues that are incorporated into mycobactin. From the unfinished M. smegmatis sequence obtained from TIGR, the organization of the mycobactin gene cluster was identical to that of M. tuberculosis with the exception of mbtI and -J (Rv2386c and Rv2385, also called trpE2 and lipK [7]). Whereas mbtI and -J map next to mbtA in M. tuberculosis, these genes were not found to be...
linked in *M. smegmatis*. Overall, the proteins encoded in the mycobactin gene cluster displayed a high degree of identity between the two mycobacterial species, from a high of 75.4% for the *mbtG* orthologues to a low of 49.7% for *mbtD*.

The flanking regions from LUN8 had homology with an *M. tuberculosis* acyl carrier protein gene (*Rv1344*; 52.7% identity) and two genes (*fadD33* and *fadE14*; 62.4 and 76.6% identity, respectively) predicted to be involved in fatty acid degradation. These genes do not map to the *mbt* gene cluster. The *M. tuberculosis* acyl carrier protein had an additional 22 amino acids at its N terminus compared to that of the *M. smegmatis* orthologue. A methionine at the end of this stretch of addi-

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**FIG. 4.** Physical map of the Tn611 insertion region of *M. smegmatis* LUN9 compared to the *M. tuberculosis* mycobactin biosynthetic cluster from nucleotides 2680005 to 2656218. Solid arrows indicate open reading frames for the respective genes. The transposon insertion is shown in the opposite orientation from that in Fig. 5 and, in contrast to the LUN8 insertion, the ISa element was duplicated instead of the ISb element.

**FIG. 5.** Physical map of the Tn611 insertion region of *M. smegmatis* LUN8 compared to the *Rv1344* (*acp*)/*fadD33*/*fadE14* sequence from nucleotides 1508968 to 1512003 of *M. tuberculosis*. The Tn611 insertion location in LUN8 is indicated. Solid arrows indicate open reading frames for the respective genes. The dashed nucleotide line for *M. smegmatis* represents partially sequenced DNA. *Pst*I digestion hybridization fragments are shown at the bottom of the figure.
DISCUSSION

Mycobactin is an almost ubiquitously occurring siderophore in both pathogenic and saprophytic mycobacteria. The saprophytic forms produce a second siderophore called exochelin (32), but loss of exochelin production did not significantly diminish the MIC of the iron chelator EDDA for *M. smegmatis* exochelin mutants. However, the necessity for mycobactin in mycobacterial iron metabolism was evidenced by the fivefold decrease in the MIC of EDDA for the mycobactin mutant strains LUN8 and LUN9. The reason for the sensitivity of the mycobactin mutants to iron restriction is unknown.

Analysis of cloned DNA sequences indicated that the regions flanking the transposon insertion in the mycobactin mutant strain LUN9 had homology with mycobactin genes *mbtD* and *mbtE* of *M. tuberculosis*. MbtD belongs to the polyketide synthase superfamily of proteins that contain consensus sites for posttranslational phosphopantetheinylation and may be involved in acyl group transfer (8). MbtE shares homology with nonribosomal peptide synthetases and has a single amino acid adenylation domain that probably activates the lysine residues that are incorporated into mycobactin. Our results are in agreement with those of De Voss et al. (11), who showed that disruption of the *M. tuberculosis* *mbt* gene cluster prevented synthesis of the mycobactin core.

The regions flanking the transposon insertion in strain LUN8 have homology with *M. tuberculosis* genes encoding an acyl carrier protein (*Rv1344*), an acyl-CoA synthase (*fadD33*), and an acyl-CoA dehydrogenase (*fadE14*). These genes, which are not located in the reported mycobactin gene cluster (7), appeared to be necessary for wild-type mycobactin production, an observation not previously reported. The preliminary NMR studies of the mycobactin made by LUN8 suggested that in *M. smegmatis* the *acp/fadD/fadE* gene cluster is involved in biosynthesis of the aliphatic side chains attached to the mycobactin nucleus. Because correct side chain synthesis cannot be accomplished in strain LUN8, it produced only a small amount of a mycobactin with an altered side chain. FadD and FadE are predicted, based on sequence, to be involved in lipid degradation (7); these enzymes could initiate modification of a preexisting lipid with subsequent attachment of the lipid to an acyl carrier protein with final transfer to the mycobactin core by an as-yet-to-be-identified acyltransferase (8, 10). Alternatively, the FadD and FadE enzymes could modify the mycobactin side chains after they have been attached by the acyltransferase.

An examination of the putative regulatory region of the *M. smegmatis* *acp* gene revealed a consensus “IdeR box,” suggesting a role for the iron-dependent regulatory protein IdeR (38) in the transcriptional control of these genes. Moreover, Rodriguez et al. (36) recently demonstrated by microarray analysis that the expression of the *M. tuberculosis Rv1344, fadD33*, and *fadE14* genes was iron and IdeR repressible. The translational termination and initiation codons for *acp/fadD* and *fadD/fadE* were predicted to overlap. These observations and the complementation data from LUN8 transformed with pBLT1344 and its subclones suggest that *acp-fadD-fadE* comprise an operon with a single polycistrionic mRNA under control of the IdeR iron-dependent regulator, making expression of the operon responsive to iron restriction.

Whereas the wild-type *M. smegmatis* LR222 genes provided the missing function(s) to strain LUN8, transformation of this strain with the *M. tuberculosis* orthologues failed to restore wild-type mycobactin synthesis. Although there is a high degree of homology between the *M. tuberculosis* and the *M. smegmatis* *acp/fadD/fadE* genes, the lack of complementation of LUN8 by the corresponding *M. tuberculosis* genes may not be surprising. An explanation may be that the FadD and FadE enzymes from the two species recognize different lipids. Moreover, the aliphatic side chains of the mycobactins are highly variable and species specific (44). *M. smegmatis* may not produce the particular lipid that is recognized by *M. tuberculosis* FadD and/or FadE. Wheeler and Ratledge (48) proposed that in vivo-grown *M. tuberculosis* makes use of the variety and quantity of lipids available within mammalian cells and the tubercle. The many FadD and FadE alleles (36 of each) encoded by the *M. tuberculosis* genome (7) would suggest a high degree of specificity of each enzyme for its substrate. Various species-specific genes may be used for synthesis and attachment of the variable side chains of mycobactin.

An alternative explanation for the lack of complementation by the *M. tuberculosis* genes could simply be that the *M. smegmatis* RNA polymerase does not efficiently initiate transcription from the *M. tuberculosis* promoter. Although the recognition of promoter sequences appears somewhat conserved among mycobacteria (28), differences in gene expression of cloned mycobacterial DNA have been reported when genes from one species are introduced into another (24, 46). Moreover, Mulder et al. (28) suggested that the validity of studying *M. tuberculosis* gene expression in other mycobacterial hosts may depend on the particular promoter in question.

A significant amount of evidence has accumulated demonstrating that two of the microbial processes that contribute to *M. tuberculosis* infection are iron acquisition and lipid metabolism (11, 12, 25, 34, 35, 36, 43). *M. tuberculosis* strains that are defective in mycobactin synthesis (11) or express a constitut
tively active iron repressor (25) are attenuated for virulence. Differential display was used to show that fadD33 is under-
expressed in the attenuated strain M. tuberculosis H37Rv (35), and strains that are defective in fadD33 expression are inhib-
ited for growth in the liver of BALB/c mice (34). Here we have demonstrated that these two processes, iron acquisition and
lipid metabolism, intersect. We have shown that a mutation in the M. smegmatis orthologue of fadD33 disrupts production of
mycobactin, resulting in a molecule with an altered side chain. In the pathogenic mycobacteria, the fad genes also may have
essential functions in both lipid metabolism and production of mycobactin, a necessary component of the iron acquisition
process.

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