Investigating the Function of the Putative Mycolic Acid Methyltransferase UmaA

**DIVERGENCE BETWEEN THE MYCOBACTERIUM SMEGMATIS AND MYCOBACTERIUM TUBERCULOSIS PROTEINS**

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Françoise Laval†§, Ruth Haitez†, Farahnaz Movahedzadeh†‡, Anne Lemassu†§, Chinn Yi Wong*, Neil Stoker†‡, Helen Billman-Jacobe‡‡, and Mamadou Daffé‡§

*From the †Institut de Pharmacologie et Biologie Structurale (IPBS), Université Paul Sabatier (Toulouse III), 205 route de Narbonne, 31077 Toulouse Cedex, France, the ‡Department Mécanismes Moléculaires des Infections Mycobactériennes, Institut de Pharmacologie et Biologie Structurale (IPBS), Centre National de la Recherche Scientifique (Unité Mixte de Recherche 5089), 205 route de Narbonne, 31077 Toulouse Cedex, France, the ¶Department of Pathology and Infectious Diseases, Royal Veterinary College, Royal College Street, London NW1 0TU, United Kingdom.

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1 Present address: Inst. for Tuberculosis Research College of Pharmacy, Rm 412, University of Illinois at Chicago, 833 S. Wood St. Chicago, Illinois 60612-7231.

2 Both authors contributed equally to the work.

3 To whom correspondence should be addressed. Tel.: 33-561-175-569; Fax: 33-561-175-580; E-mail: daffe@ipbs.fr.

Mycolic acids are major and specific lipid components of the cell envelope of mycobacteria that include the causative agents of tuberculosis and leprosy, Mycobacterium tuberculosis and Mycobacterium leprae, respectively. Subtle structural variations that are known to be crucial for both their virulence and the permeability of their cell envelope occur in mycolic acids. Among these are the introduction of cyclopentyl groups and methyl branches by mycolic acid S-adenosylmethionine-dependent methyltransferases (MA-MTs). While the functions of seven of the M. tuberculosis MA-MTs have been either established or strongly presumed nothing is known of the roles of the remaining umaA gene product and those of M. smegmatis MA-MTs. Mutants of the M. tuberculosis umaA gene and its putative M. smegmatis orthologue, MSMEG0913, were created. The lipid extracts of the resulting mutants were analyzed in detail using a combination of analytical techniques such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and proton nuclear magnetic resonance spectroscopy, and chemical degradation methods. The M. smegmatis mutants no longer synthesized subtypes of mycolates containing a methyl branch adjacent to either trans cyclopropyl group or trans double bond at the “proximal” position of both α- and epoxy-mycolates. Complementation with MSMEG0913, but not with umaA, fully restored the wild-type phenotype in M. smegmatis. Consistently, no modification was observed in the structures of mycolic acids produced by the M. tuberculosis umaA mutant. These data proved that despite their synteny and high similarity umaA and MSMEG0913 are not functionally orthologous.

Mycolic acids, α-branched β-hydroxylated long chain fatty acids, represent major and very specific constituents of the lipid-rich envelope of mycobacteria such as Mycobacterium tuberculosis, the causative agent of tuberculosis, and play an important structural role in the envelope architecture (1–4). For instance, mycolic acids bound to the cell wall arabinogalactan are organized with other lipids to form an outer barrier with an extremely low fluidity that confers an exceptional low permeability to mycobacteria and may explain their intrinsic resistance to many antibiotics (5). Other natural constituents containing mycolic acids, such as trehalose mycolates, are known to be implicated in numerous biological functions related both to the physiology and virulence of M. tuberculosis (3). Furthermore, their biosynthesis is the only clearly identified target inhibited by the major antitubercular drug, isoniazid (2, 3, 6). Therefore, deciphering the biosynthesis pathway leading to mycolates still represents a major objective.

Mycolic acids are found in mycobacterial species as a mixture of structurally related molecules that differ primarily from one another by the nature of the chemical groups at the so-called “proximal” and “distal” positions of their main (meromycolic) chain (Fig. 1). In M. tuberculosis and phylogenetically close species, as well as in several slow growing mycobacteria, three types of mycolates commonly occur (7, 8). The least polar α-myo- lates of M. tuberculosis consist of C_{76–82} fatty acids (9) and contain two cis cyclopropyl groups. The more polar mycolates are composed of C_{80–82} fatty acids (9) and contain both a cis or trans (with a methyl group on the vicinal carbon atom) cyclopropyl group at the proximal position, and a methoxy- or keto-group (with a methyl group on the vicinal carbon atom) at the distal position (Fig. 1A). Mutations resulting in either the loss or the modification of these chemical functions profoundly alter the perme-
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| Alpha | Methoxy | Keto |
|-------|---------|------|
| α     | D       | P    |
|       | CH₃(CH₆H₅)₃CH₂CH₂(CH₆H₅)₃CH₂(α) | CH₂ |
|       | /      | OH COOH |
|       | /      |        |
|       |       |        |

| Epoxy |        |
|-------|--------|
| α'    |        |
|       |        |
|       |        |

FIGURE 1. Structures of the major mycolic acids of M. tuberculosis (A) and M. smegmatis (B). The cyclopropyl ring at the proximal (P) position (relative to the carboxyl group) in methoxy- and keto-mycolates of M. tuberculosis, and the double bonds in mycolates of M. smegmatis may have a methyl branch at the adjacent position, in which case they exhibit a trans configuration. D indicates the distal position (relative to the carboxyl group) of the meromycolic chain. The main values of m₁ and m₃ are 15, 17, and 19; those of m₂ are 14 and 16 when no methyl branch occurred in the molecules, and 13, 15, and 17 in compounds containing a methyl branch. The values of m₁ and m₃ are higher in methoxy- and keto-mycolates than in α- and epoxy-mycolates (see Ref. 9).

Ability of the cell envelope to solutes and severely affect the virulence and pathogenicity of the mutant strains in experimental infections (10–14). Accordingly, the enzymatic systems that introduce the chemical modifications in the mycolic acid chain, mycolic acid S-adenosylmethionine-dependent methyltransferases (MA-MTs), merit special attention as these discrete structural changes may be of crucial biological importance. In M. smegmatis and other rapid growing mycobacterial species, which are generally non pathogens, cis and trans double bonds are found in place of cyclopropyl groups in both α-mycolates and related oxygenated molecules (Fig. 1B).

M. tuberculosis has eight predicted MA-MTs (cmaA1–2, mmaA1–4, pcaA, and umaA) (15). These map on the genome with mmaA1–4 together, cmaA1–2 separately and pcaA and umaA together and convergently transcribed. Of the eight MA-MT genes, four have been unambiguously characterized through gene inactivation. Characterization of the resulting mutant strains has shown that the cmaA2 gene encodes a methyltransferase required for the synthesis of trans cyclopropyl at the proximal position of methoxy- and keto-mycolates in M. tuberculosis (16) whereas the mmaA2 gene catalyzes the introduction of a cis cyclopropyl group at both the distal position of α-mycolates and proximal position of methoxy- and keto-mycolates (17). The umaA4 gene (18), also called imaA, has been shown to be necessary for the synthesis of both keto- and methoxy-mycocard acids (10) whereas the umaA2 (Rv0470c) gene has been shown to encode the methyltransferase that is responsible for the cyclopropagation of the double bond at the proximal position of α-mycolates in M. tuberculosis and, accordingly, named pcaA (12). PcaA has been shown to be required for both the cording of the tubercle bacillus and its long term virulence in mice (12) and is absent from the genome of the non pathogenic M. smegmatis. For the remaining candidates, the cmaA1, mmaA1, and mmaA3 genes from M. tuberculosis have been overexpressed in a surrogate host, M. smegmatis, and the three putative MA-MTs have been shown to function as methyltransferases (18, 19) although subsequent inactivation of cmaA1 in M. tuberculosis has led to no phenotypical change (17). In contrast, nothing is known on the reaction catalyzed by the remaining umaA (Mt-umaA) gene product.

M. smegmatis has at least seven MA-MT paralogues; two genes are present in the mmaA1–4 region, and one in the umaA-pcaA region in the same orientation as Mt-umaA. The cmaA1 and cmaA2 regions are not present in M. smegmatis although other genes in these regions are conserved. M. smegmatis has four additional paralogues. All seven proteins are similar (73–77% similarity) and the conserved gene arrangement in some loci suggests that some of the M. tuberculosis and M. smegmatis genes may be or may have been orthologues. Mt-umaA is most similar to M. smegmatis MSMEG0913 in terms of homology (85% similarity) and is also syntenic. The present study was undertaken in order to determine whether the two genes are orthologues and to investigate their functions.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—M. tuberculosis and M. smegmatis strains were cultured on Middlebrook 7H10 agar. For liquid cultures, Middlebrook 7H9 broth (Difco) and 0.05% Tween 80 was used with 10% oleic acid-albumin-dextrose-catalase (OADC) supplement (Becton Dickinson) for spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
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M. tuberculosis strains. M. tuberculosis cultures contained hygromycin (Hyg) (100 μg ml⁻¹), kanamycin (Kan) (20 μg ml⁻¹), gentamicin (10 μg ml⁻¹), and X-gal (50 μg ml⁻¹) and M. smegmatis cultures contained streptomycin (Str), Hyg or Kan (20 μg ml⁻¹) where required. All cultures were incubated at 37 °C and with shaking for liquid cultures.

Isolation of a M. smegmatis MSMEG0913 Mutants—A library of Tn611 (20) mutants of M. smegmatis was screened using inverse PCR and amplification of the chromosomal sequence flanking the site of insertion with the Tn611-specific oligonucleotide primers (Supplemental Table S1) on a genomic DNA template that had been digested with RsaI and then self-ligated. One mutant, Myco69, had a Tn611 insertion 117-bp upstream of the MSMEG0913 start codon.

A mutant of M. smegmatis where the MSMEG0913 open reading frame (ORF) was disrupted was made by inserting a kan resistance gene into the ORF. A 2.5-kb fragment containing MSMEG0913 was PCR-amplified from M. smegmatis mc²155 using oligonucleotide primers 436 and 437 (Supplemental Table S1) and the PCR product was cloned into pHBLUNTII-TOPO (Invitrogen). The insert was excised from this plasmid with XbaI and cloned into the XbaI site of the sacB-str suicide vector, pHBJ428 (21). The resulting plasmid, pHBJ474 was linearized with NcoI within the MSMEG0913 ORF and a kan resistance cassette was inserted to disrupt the ORF. The kan cassette was in the opposite orientation to the MSMEG0913 ORF. The resulting plasmid, pHBJ489, was transformed into M. smegmatis and transformants were plated onto Middlebrook 7H10 agar containing either kan or str. A single crossover strain, Myco735 was isolated. Disruption of MSMEG0913 was confirmed by Southern blot analysis. Both MSMEG0913 mutants had the same colony morphology and growth characteristics as M. smegmatis mc²155 when grown on Middlebrook media.

Construction of a M. tuberculosis umaA Mutant—Mutagenesis was carried out essentially as described previously (22). The coding sequence of M. tuberculosis H37Rv umaA with flanking DNA, 978-bp upstream and 977-bp downstream of the gene was amplified by PCR using primers umaA1 and umaA2 (Supplemental Table S1) in a reaction with 10% v/v Me₂SO. The PCR product was phosphorylated using polynucleotide kinase and cloned into the PmlI site of plasmid p2NIL, producing pFM173. The umaA1 gene was eliminated by inverse PCR, using umaAR1 and umaAR2 (Supplemental Table S1) in a reaction with 5% v/v Me₂SO. The PCR product consisting of the vector and the regions originally flanking umaA was phosphorylated using polynucleotide kinase followed by re-ligation (pFM183). Following insertion of a gene cassette carrying lacZ and sacB from pGOAL19 into the vector PacI site to produce pFM194, the DNA was introduced into M. tuberculosis H37Rv by electroporation. Cells carrying single-crossovers were isolated by selection for blue hygR kanR colonies on Middlebrook 7H11 agar containing OADC supplement (Difco). One single crossover colony was plated onto agar containing 2% w/v sucrose to isolate bacteria with a double crossover event.

Mutant strains were selected by PCR and confirmed by Southern analysis (data not shown). One of these (FAME89) was analyzed further.

Complementation of the M. smegmatis MSMEG0913 Mutant—The mutant, Myco735, was complemented with MSMEG0913, which had been amplified by PCR using primers 420 and 421 (Supplemental Table S1) and cloned as a BamH1/HindIII fragment into pVV16 creating strain Myco740. Myco735 with a vector control was named Myco742. This construct contained the entire MSMEG0913 gene, and part of each flanking gene. Mt-umaA and Mt-mmaA1 genes were each PCR-amplified (primer 604 and 605 and 612 and 613, respectively (Supplemental Table S1) and cloned into pCR-BluntII-TOPO then subcloned individually as a BamH1-HindIII fragments into pVV16, which had been digested with the same enzymes producing pHBJ616 (Mt-umaA) and pHBJ624 (Mt-mmaA1). A second Mt-umaA complementation construct was made in a version of pVV16 in which the hsp60 promoter had been removed by Xba1 digestion. The cloned fragment of M. tuberculosis DNA contained the 5’-end of Mt-uma through to 1-kb upstream of the gene, including the natural promoter. All complementation constructs expressed recombinant proteins with C-terminal hexahistidine epitopes. The expression of the proteins in M. smegmatis strains was confirmed by Western blotting of cell lysates with anti-hexahistidine monoclonal antibodies (data not shown).

Purification of Mycolic Acids—Whole cells or bacterial residues obtained after lipid extraction with organic solvents (10) were saponified by a mixture of 40% KOH and methoxyethanol (1:7, v/v) at 110 °C for 3 h in a screw-capped tube. After acidification, fatty acids were extracted with diethyl ether and methylated with an ethereal solution of diazomethane (7). The mycolate patterns of the strains were determined by analytical thin-layer chromatography (TLC) on Silica Gel 60 (Silica Gel 60 Macherey-Nagel) using either eluent A (dichloromethane) or eluent C (petroleum ether/diethyl ether; 9:1, v/v, five runs). Revelation of lipid spots was performed by spraying the plates with molybdophosphoric acid (10% in ethanol), followed by charring. The different classes of mycolates were separated by chromatography on a Florisil column irrigated with increasing concentrations of diethyl ether (0, 10, 20, 30, and 50%, v/v) in petroleum ether and purification was achieved by preparative TLC using eluent A (9). To search for the presence of ethylenic compounds, the various purified mycolate types were analyzed and fractionated on AgNO₃-impregnated silica gel TLC plates developed with eluent A (CHCl₃/CH₃OH). The various purified types and subtypes of mycolates were quantified by weighing.

Degradative Techniques—Cleavage of double bonds was performed by permanganate-periodate oxidation (23) at 30 °C in tertiary butanol as solvent. The acids obtained from the oxidative cleavages were methylated and purified on preparative TLC with dichloromethane as eluent.

Miscellaneous Analytical Techniques—¹H NMR spectra of purified mycolic acid methyl esters were obtained in CDCCl₃ (100% D) using a Bruker AMX-500 spectrometer at 298 K. Chemical shifts values (in p.p.m.) were relative to the internal CHCl₃ resonance (at 7.27 ppm).
MALDI-MS detection of purified samples in reflectron mode was performed on a 4700 Proteomics Analyzer (Applied Bio-systems, Voyager DE-STR, Framingham, MA) equipped with a Nd :YAG laser (355 nm) operating by pulses of 500 ps with a frequency of 200 Hz. 2500 shots were accumulated in positive ion mode and MS data were acquired using the instrument default calibration. Mycolates samples were dissolved in chloroform, at the concentration of 1 mM, and were directly spotted on the target plate as 0.5-μl droplets, followed by the addition of 0.5 μl of matrix solution. Samples were allowed to crystallize at room temperature. The matrix used was 2,5-dihydroxybenzoic acid (10 mg/ml) in CHCl3/CH3OH (1:1, v/v).

Gas chromatography (GC) of fatty acid methyl esters derived either from the saponification of whole cells or from degradation of purified mycolates was performed on a Hewlett-Packard 5890 series II apparatus equipped with an OV1 capillary column (0.30 mm × 25 m) using helium gas. The temperature separation program involved an increase from 100 to 300 °C at the rate of 5 °C/min, followed by 10 min at 300 °C.

RESULTS

M. smegmatis MSMEG0913 Mutants Have Subtle Modifications in the Structures of Mycolates—Lipids of M. smegmatis mc2155 and mutants Myco69 and Myco735 were analyzed as described. All the strains contained the same types of major extractable lipids, which included triacylglycerol, glycerol mycolate, trehalose mono- and di-mycolates, and phospholipids (data not shown). M. smegmatis mc2155, Myco69, and Myco735, had comparable contents in extractable lipids (20% of the bacterial dry mass) and in wall-linked mycolates (9% of the bacterial residue dry mass). They also showed identical C16–C24 fatty acid methyl ester profiles on GC and types of mycolates on silica gel TLC, namely α-, α′- and epoxy-mycolates (Fig. 2A). To determine whether the inactivation of the

MSMEG0913 resulted in the absence methyl branches in the mycolates of the mutant strains, the various types of mycolates of the parental and mutant strains of M. smegmatis were purified and comparatively analyzed by MALDI-TOF mass spectrometry (9) and 1H NMR spectroscopy (24). The mass and NMR spectra of purified mycolates of the Myco69 and Myco735 were superimposable but different from those of the mc2155. As a consequence, only the data from Myco735 will be presented hereafter.

Although the mass spectra of the α-mycolates of the wild-type and the mutant contained pseudomolecular ion (M+Na)+ peaks assignable to C74 to C82 α-mycolic acid methyl esters the major peaks were observed at 1160 and 1188 m/z (corresponding to C77 and C79, respectively) in the mass spectra of the mutant strains (Fig. 3A). Similarly, the major pseudomolecular ion peaks assignable to epomycolates were observed at 1204 and 1218 m/z (corresponding to C79 and C80, respectively) in the mass spectra of mc2155 whereas they were seen at 1176 m/z (C79) and 1204 m/z (C79) in the mass spectra of the mutant strains (Fig. 3B). These differences were independent from both the temperature and the phase of growth of the strains and suggested that modifications have occurred in the structures of both α- and epoxy-mycolates of Myco69 and Myco735. In contrast, comparable intense pseudomolecular ion peaks attributable to C63 and C64 α′-mycolic acid methyl esters, at 951.92 and 979.95 m/z, were observed in the mass spectra of the purified mycolates from all strains (data not shown), confirming the absence of methyl branch in α′-mycolates (3).

Purification and Characterization of the Subtypes of α-Mycolates from M. smegmatis—To further structurally characterize the mycolates from the mutant strains and those of the parent the various purified mycolates were further analyzed by AgNO3-impregnated TLC (Fig. 2B), a layer that is known to have an affinity for cis-ethylenic bonds. A single spot was detected for α′-mycolates from mc2155 and the mutants, as expected (data not shown). In contrast, four major α-mycolate spots, called αy to αz and a minor αx form, were detected and isolated by preparative AgNO3-impregnated TLC from mc2155 whereas three of them, αx, αy, and αz were observed in the mutants (Fig. 2B). The αx-mycolates, present in all the strains, exhibited the same mobility on AgNO3-impregnated TLC as the dicyclopropanated mycolic acid methyl esters from the control strain while the αy- and αz-mycolates had a migration consistent with the presence of one cyclopropyl group and one double bond of trans and cis configuration, respectively.

FIGURE 2. Thin-layer chromatography of methyl esters of mycolic acids from M. smegmatis (A–C) and M. tuberculosis (D). A and D, direct phase chromatography of the mixtures of mycolates on silica gels: α, α′-mycolates; α, α′-mycolates; E, epomycolates; M, methoxymycolates; K, ketomycolates. B and C, argentation chromatography of purified α-mycolates and epomycolates, respectively. Solvents: dichloromethane (A–C) and petroleum ether/diethylether 9:1 (v/v, five runs) (D). Visualization: molybdophosphoric acid, followed by charring. Panels A–C, lanes 1, mc2155; 2, MSMEG0913 mutant (Myco735); 3, Myco735 complemented with the MSMEG0913 (Myco740). Panel D, lane 1′, M. tuberculosis H37Rv; 2′, FAME89 umaA3 mutant strain.

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RESULTS

M. smegmatis MSMEG0913 Mutants Have Subtle Modifications in the Structures of Mycolates—Lipids of M. smegmatis mc2155 and mutants Myco69 and Myco735 were analyzed as described. All the strains contained the same types of major extractable lipids, which included triacylglycerol, glycerol mycolate, trehalose mono- and di-mycolates, and phospholipids (data not shown). M. smegmatis mc2155, Myco69, and Myco735, had comparable contents in extractable lipids (20% of the bacterial dry mass) and in wall-linked mycolates (9% of the bacterial residue dry mass). They also showed identical C16–C24 fatty acid methyl ester profiles on GC and types of mycolates on silica gel TLC, namely α-, α′- and epoxy-mycolates (Fig. 2A). To determine whether the inactivation of the
more polar α₁- and α₂-mycolates may correspond to diethyl-
enic molecules. Analysis of the MALDI-TOF mass spectra of
the various purified compounds showed that the α₁-mycolates
from mc²155 were composed of C₇₀, C₈₀, C₈₁, and C₈₃ mole-
cules. In contrast, the α₁-mycolates from the mutant Myco735
were devoid of molecules containing odd number of carbon
(Table 1). α₂-mycolates from mc²155, a subclass of mycolates
that were absent from the mutants, consisted of C₇₀, C₈₀, and
C₈₂ constituents whereas α₁- and α₂-mycolates were mainly
composed of C₇₇ and C₇₉ molecules (Table 1). α₃-mycolates
that was abundantly produced by the mutant consisted of C₇₆,
C₇₉, C₈₀, and C₈₂. To discriminate between the various structural
possibilities for the different α-mycolates, especially in terms
of contents in cyclopropyl groups and/or double bonds, the puri-
ified subclasses of α-mycolates were examined by NMR
spectroscopy.

Analysis of the different types of purified mycolates by ¹H
NMR showed that all the spectra contained the huge signal
attributed to the isolated methylene proton resonances (at 1.29
ppm, broad signal), signals due to terminal methyl groups (at
0.85 ppm, triplet), methoxyl from the carboxyl methyl ester (at
3.71 ppm, singlet), methine located at position C-2 (at 2.50
ppm, multiplet). In addition, the spectrum of α₁-mycolates
from mc²155 (Fig. 4a) contained signal resonances of both cis-
(at −0.33, +0.57 and +0.64 ppm) and trans- (at 0.15 and 0.47
ppm) cyclopropyl protons and devoid of signal resonances
of double bond protons (at δ 5.34). This observation was consis-
tent with the data obtained by MALDI-TOF mass spectrometry
showing that these mycolates were composed of molecules containing
both odd and even numbers of car-
on. It could thus be deduced that the α₁-mycolates of mc²155 were
composed of two types of dicyclo-
propanated mycolates: (i) molecules
with an even number of carbon con-
taining only cis cyclopropyl rings
and (ii) compounds with an odd
number of carbon containing one
cis and one trans cyclopropyl
rings. As trans cyclopropyl rings
are known to have an adjacent
methyl branch (3), the latter type
of mycolates probably corresponds to
the longest C₈₁ and C₈₃ molecules
(Table 1). In agreement with this
hypothesis, the ¹H NMR spectrum
of α₁-mycolates from the mutant
strains contained signals attributa-
table to resonances of cis-cyclopropyl
protons with only traces of trans
molecules (Fig. 4b). This indicated

![Figure 3](https://example.com/figure3.png)

**FIGURE 3.** MALDI-TOF mass spectra of the purified α- (A) and epoxy-mycolates (B) of *M. smegmatis*
mc²155 and MSMEG0913 mutant (Myco735). Values indicate the masses of the sodium adducts (M+23).

| Table 1 |
|-------------------------------|
| **Comparative data from TLC, MALDI-TOF MS and NMR analyses of subtypes of α- and epoxy-mycolates from *M. smegmatis* mc²155 and Myco735 mutant** |
| **Rf** | **Ratio** | **Carbon number deduced from the analysis of MALDI-TOF MS** | **Chemical functions present in the mero-chain as deduced from ¹H NMR** |
| α₁ mc²155 | 0.50 | 1.0 | C₇₈, C₈₀, C₈₁, C₈₃ | cis CP + trans CP **a** |
| a₁ Myco735 | 0.48 | 1.0 | C₇₈, C₈₀, C₈₂ | cis CP |
| a₂ mc²155 | 0.26 | 2.2 | C₇₈, C₈₀, C₈₂ | cis CP + trans DB |
| a₂ Myco735 | - | - | - | - |
| a₃ mc²155 | 0.13 | 1.3 | C₇₅, C₇₇, C₇₉ | cis CP + cis DB |
| a₃ Myco735 | 0.13 | 2.5 | C₇₇, C₇₉, C₈₁ | cis CP + cis DB |
| a₄ mc²155 | 0.06 | 3.0 | C₇₇, C₇₉ | cis DB + trans DB |
| a₄ Myco735 | - | - | - | - |
| a₅ mc²155 | 0.03 | 0.6 | C₇₆, C₇₈, C₈₀ | cis DB |
| a₅ Myco735 | 0.03 | 7.5 | C₇₆, C₇₈, C₈₀ | cis DB |
| E₁ mc²155 | 0.26 | 1.0 | C₇₈, C₈₀, C₈₁, C₈₃ | trans E + cis CP |
| E₁ Myco735 | 0.26 | 1.0 | C₇₈, C₈₀, C₈₂ | trans E + cis CP |
| E₂ mc²155 | 0.13 | 1.0 | C₇₈, C₈₀ | trans E + cis DB |
| E₂ Myco735 | - | - | - | - |
| E₃ mc²155 | 0.06 | 1.5 | C₇₇, C₇₉ | trans E + cis DB |
| E₃ Myco735 | 0.06 | 2.0 | C₇₇, C₇₉ | trans E + cis DB |

**a** The amounts of the different subtypes of mycolates were determined by weighing the purified compounds; the amounts of a₁ and E₁ from the mc²155 strain were arbitrary fixed to 1.

**b** The number of carbon for the major component is indicated in bold.

**c** cis and trans indicated the configuration of double bonds (DB) and cyclopropyl (CP) groups in the different subtypes of α- and epoxy-mycolates (E).
that they consisted almost exclusively of dicyclopropanated mycolates with cis configuration and with an even number of carbon, $C_{30}$ and $C_{32}$ molecules (Table 1).

The $^1$H NMR spectrum of $\alpha_\gamma$-mycolates from mc$^{155}$ (Fig. 4c) contained signals attributable to resonances of cis-cyclopropyl protons and trans double bond protons ($\delta$ 5.32) whereas that of $\alpha_\delta$-mycolates from both strains contained signals assignable to resonances of cis double bond and cis cyclopropyl protons (Fig. 4d). Consistently, $\alpha_\gamma$-mycolates exhibited an even number of carbon (Table 1), as trans double bonds in mycolates are known to have a methyl branch at the adjacent position (3). The $^1$H NMR spectrum of $\alpha_\delta$-mycolates that also typified the parental strain was devoid of resonances of cyclopropyl protons but contained resonances attributed to both cis and trans ethylenic protons (Fig. 4e). Similarly, the $^1$H NMR spectrum of $\alpha_\delta$-mycolates, which were prominent in the mutant strain, was also devoid of resonances of cyclopropyl protons but, in contrast to that of $\alpha_\gamma$-mycolates from mc$^{155}$, contained resonances attributed to only cis ethylenic protons (Fig. 4f). This observation was consistent with the even number of carbons of $\alpha_\delta$-mycolates (Table 1) and the absence of a methyl branch in mycolates of the mutant, as cis double bonds are known to be devoid of a methyl branch at the adjacent position (3). It was thus concluded that the disruption of the MSMEG0913 of M. smegmatis resulted in the production of $\alpha$-mycolates with cis cyclopropyl groups and/or cis double bonds and the disappearance of both trans cyclopropyl groups (found in $\alpha_\gamma$-mycolates) and trans double bonds (found in $\alpha_\delta$- and $\alpha_\delta$-mycolates) of the parental strain (Table 1).

**Purification and Analysis of the Subtypes of Epoxymycolates**

The various classes of epoxymycolates from the mc$^{155}$ and Myco735 were analyzed by AgNO$_3$-impregnated TLC (Fig. 2C). This showed that mc$^{155}$ contained three major spots, called E$_1$ to E$_3$, whereas the mutants contained only two well visible mycolate spots, E$_1$ and E$_3$. The E$_1$-mycolates from the parental strain consisted of $C_{78}$, $C_{80}$, $C_{81}$, $C_{82}$, and $C_{83}$ molecules whereas those of the mutants were composed of molecules containing an even number of carbon only (Table 1). E$_2$-mycolates that typified mc$^{155}$ consisted in $C_{78}$ and $C_{80}$. E$_1$-mycolates from the parental and mutant strains were composed in majority of $C_{77}$ and $C_{79}$ molecules.

Analysis of the purified subclasses of epoxymycolates by $^1$H NMR (Fig. 5) showed that all the spectra contained, in addition to the common signals shared by all the mycolates (see above), the signal resonance assigned to the methine protons of a trans epoxy ring at 2.65 ppm (25–27), the resonances of cis-epoxymycolates being expected at 2.90 ppm (24). The resonances of the protons of the methyl branch adjacent to the trans epoxy group were also observed as a doublet at 1.10 ppm (25–27).

The $^1$H NMR spectrum of E$_1$-mycolates from mc$^{155}$ contained also signal resonances of both cis- and trans-cyclopropyl protons and devoid of resonances of double bond protons (Fig. 5a); the corresponding $^1$H NMR spectrum of the mutants contained signal resonances of cis cyclopropyl protons with only traces of trans molecules (Fig. 5b). This observation indicated that mc$^{155}$ produced two populations of trans-epoxy-mono-cyclopropanated mycolates: (i) $C_{78}$ and $C_{80}$ cis cyclopropanated molecules and $C_{81}$ and $C_{83}$ trans cyclopropanated substances (Table 1). The spectrum of E$_2$-mycolates from mc$^{155}$ contained no signal resonances attributable to cyclopropyl protons (Fig. 5c); instead, it showed signals attributable to resonances of...
trans double bond protons (δ 5.32); in addition, signals of the proton resonances of the methyl branch adjacent to trans double bonds were observed at 0.94 ppm (Fig. 5c). The 1H NMR spectrum of E₃-mycolates from all the strains contained signals assignable to resonances of cis double bond protons (at 5.34 ppm) and devoid of resonances of protons of both cyclopropyl and methyl branch adjacent to double bond (Fig. 5d). It was thus concluded that the disruption of the MSMEG0913 resulted in the production of cyclopropanated and ethylenic epoxy mycolates and the disappearance of the methyl branch that occurs at the proximal position of trans cyclopropyl ring (found in a fraction of E₁-mycolates) and trans double bond (found in E₂-mycolates) from the parental strain.

Localization of Double Bonds in Mycolates—Data on the location of the methyl branches adjacent to trans double bonds in mycolates, the production of which was abolished in the Myco69 and Myco735 mutants, were obtained by oxidative cleavage (23), a method known to cleave ethylenic bonds of mycolates and to yield mono- and/or di-carboxylic acids (11, 24). When the α₃-mycolates from Myco735 were oxidized and the resulting fatty acids esterified, purified, and analyzed by TLC, three spots were detected; their migrations corresponded to those of esters of non-hydroxylated fatty acid (e.g. C₁₈), of ω-carboxylic acid (e.g. C₁₄) and of hydroxylated ω-carboxylic acid (data not shown). GC and GC/MS analyses of the non-hydroxylated fatty acid methyl esters identified them as heptadecanoic, nonadecanoic, and eicosanoic acid methyl esters, establishing the values of m₁ as 21 and allowing the determination of m₃ as 14. The remaining long-chain hydroxylated diester cleavage products of mycolates were purified and analyzed by MALDI-TOF mass spectrometry, comparatively to the ω-carboxylic acid methyl ester of similar structure isolated from M. phlei (9). Analysis of the MALDI-TOF mass spectra of the long-chain diacid methyl esters showed pseudomolecular peaks at 703, 731, and 759 m/z. Pyrolytic cleavage of these hydroxylated ω-carboxylic acid methyl esters in the GC apparatus liberated a tetracosanoic acid methyl ester, establishing the value of m₄ as 21 and allowing the determination of m₃ as 15, 17, and 19.

Inactivation of umaA in M. tuberculosis Did Not Affect the Mycolic Acid Composition—M. tuberculosis CDC1551 has a frameshift mutation in umaA; therefore it is a naturally occurring umaA mutant. Analysis of its mycolates by MALDI-TOF mass spectrometry showed no significant differences, compared with those of M. tuberculosis H₃₇Rv (data not shown), suggesting that umaA is not critical for mycolic acid synthesis in this strain. Because there could be other changes in CDC1551 that account for the results observed, a umaA-deleted mutant strain of H₃₇Rv was constructed (FAME89). This mutant exhibited similar content in extractable lipids (20–21% of the bacterial dry mass) and in wall-linked mycolates (10% of the bacterial residue dry mass) to M. tuberculosis H₃₇Rv. No obvious difference was seen between the two strains in terms of major extractable lipids detected by TLC; these included phthiocerol dimycolates, sulfatides, triacylglycerol, glycerol mycolate, trehalose monomycolate, trehalose dimycolates, and phospholipids (data not shown). They also showed identical C₈₂–C₂₆ fatty acid methyl ester profiles on GC and types of mycolates on silica gel TLC, namely α₁, methoxy- and keto-mycolates (Fig. 2D). To determine whether the inactivation of Mt-umaA resulted in the absence of cyclopropyl groups and/or methyl branches in the mycolates of the mutant strains, the total fatty methyl esters of FAME89 and H₃₇Rv were comparatively analyzed by MALDI-TOF mass spectrometry. The mass spectra of the lipid compounds from the mutant strain were superimposable to those of the wild-type H₃₇Rv strain, notably in the region of 1100–1300 m/z, which corresponds to the masses of mycolates (9). The major pseudomolecular ion (M)⁺ (data not shown), which was attributable to methoxy- (major peaks at m/z 1146, 1174, and 1202 m/z; these corresponded to C₇₆O, C₇₉O, and C₈₀O, respectively. Two other series of peaks were attributable to keto-mycolates (major peaks at m/z 1262 for C₈₂O for C₈₃O and C₈₄O) and keto-mycolates (major peaks at m/z 1246 for C₈₂O, 1274 for C₈₄O, 1302 for C₈₆O, 1316 for C₈₇O, and 1330 for C₈₈O) were observed in the MALDI-TOF mass spectra of the fatty acid methyl esters from both strains. The lack of modification of mycolate structures in the mutant was confirmed by AgNO₃-impregnated TLC. Cells from the H₃₇Rv and FAME89 harvested in either early or late logarithmic phase exhibited identical lipid patterns, as judged by GC, TLC and mass spectrometry (data not shown). It was thus concluded that the inactivation of the umaA gene in M. tuberculosis did not result in a significant change in the mycolic acid composition.
Function of the Putative Mycolic Acid Methyltransferase UmaA

in a significant change in the structures of the mycolates of the resulting mutant strain.

Complementation of M. smegmatis Myco735 with Mt-umaA and Mt-mmaA1—Possible reasons to explain the lack of effect of the deletion of Mt-umaA from M. tuberculosis are (i) Mt-umaA is not expressed in axenic culture, (ii) Mt-umaA is redundant and another MA-MT can compensate for it, or (iii) Mt-umaA is not an orthologue of MSMEG0913. The first possibility is unlikely as transcripts of Mt-umaA have been detected under the same conditions used here, and the polypeptide has been identified in the M. tuberculosis proteome. Redundancy is a possibility; MSMEG0913 is most similar in sequence to umaA and mmaA1 of M. tuberculosis, but there is still a degree of divergence. To test hypotheses ii and iii, we cloned Mt-umaA and Mt-mmaA1 into pVV16, and transformed them into M. smegmatis mc2155 and Myco735 to test if either could complement the MSMEG0913 mutation. Expression of the M. tuberculosis genes in both M. smegmatis strains was confirmed by Western blotting of the C-terminal hexahistidine epitope. Analysis of lipids showed that neither Mt-umaA nor Mt-mmaA1 complemented Myco735. The result for Mt-umaA was surprising. Closer examination of the Mt-umaA sequence showed that there are two possible translational start codons. The codon atg2 is the annotated start of an 861-bp gene (Tuberculist), and the annotation is reasonable given the occurrence of a putative ribosome binding site just upstream. The alternative start, atg1 would add 6 amino acids to the N-terminal of UmaA. Another version of Mt-umaA was cloned to include the extra amino acids. The construct was introduced into M. smegmatis mc2155 and Myco735, expression was confirmed by Western blotting, and the lipids were analyzed as before; however, there was no functional complementation. It was thus concluded that MSMEG0913 is not the orthologue of Mt-umaA.

DISCUSSION

The lipids of M. smegmatis mc2155 were comparatively analyzed with those two MSMEG0913 mutants of M. smegmatis, Myco735 and Myco69. Application of MALDI-TOF mass spectrometry, 1H NMR spectroscopy and chemical degradation techniques to the analysis of the various purified subclasses of mycolates produced by the M. smegmatis mutants revealed new structural features of mycolates from M. smegmatis despite the numerous studies previously dedicated to these compounds since 1960 (28–30). For instance, this is the first report of the existence of trans cyclopropanated α-mycolates in the mycobacterial world. More importantly, the series of structural analyses established the function of the MSMEG0913 protein as an enzyme that adds a methyl branch to the vicinal position of both a cis double bond and cyclopropyl group located at the proximal position of both α- and epoxy-mycolic acids to yield trans homologues. The mutants lacked all semblances of trans double bond in their mycolates while producing normal amounts of both cis mono- and di-ethylenic α-mycolates, di-cis-cyclopropanated α-mycolates and mono-ethylenic and mono-cis-cyclopropanated epoxymycolates. This mycolate phenotype was specific to the absence of the MSMEG0913 gene because complementation with the wild-type gene fully restored the occurrence of both trans double bond and cyclopropane in both α- and epoxy-mycolates.

The M. smegmatis results contrasted with those obtained from analysis of M. tuberculosis Mt-umaA mutants, CDC1551 and FAME89 which had the same mycolate phenotypes as the control strain, M. tuberculosis H37Rv. No differences were observed between the lipid patterns of the mutants and those of the control strain, in terms of amounts of extractable and cell wall-linked lipids and in structures of these compounds and their constituents implying that the umaA gene is not essential for the production of the methyl branches and cyclopropyl rings in mycolates in M. tuberculosis. The redundancy of Mt-umaA appears to be shared by M. leprae that produces mycolates exhibiting the same structures as those of M. tuberculosis, i.e. dicyclopropanated α-, and (cis and trans) monocyclopropanated keto- and methoxy-mycolates (31, 32); however, the M. leprae umaA gene is predicted to be a pseudogene (33). It may be simply that another MA-MT can catalyze the reactions performed by the M. smegmatis MSMEG0913. However, despite their synteny and high homology, the failure of Mt-umaA or Mt-mmaA1 to complement the M. smegmatis mutants showed that the genes are no longer functionally orthologous.

McAdam et al. (34) showed that Tn disruption of Mt-umaA resulted in hypervirulence of M. tuberculosis in SCID mice, proving that the gene is functional in H37Rv, and inferred that altered mycolates had a role in the phenotype. Our data showing that Mt-umaA deletion had no detectable effect on mycolates in axenic culture suggest that the role of Mt-umaA may depend on environmental factors that occur in vivo. The situation with Mt-umaA may be comparable to that of Fb ps of M. tuberculosis. Although the inactivation of fb pA affected the multiplication of the mutant in macrophages (35), no differences were seen in terms of lipid content and profile between the parental and the fb pA mutant (36). Interestingly, the fb pA was shown to complement the deficiency of the fb pC mutant (36), establishing its function. In other mycobacterial species and/or models of infection, the lack of production of the UmaA protein may result in an attenuation phenotype, as reported for M. paratuberculosis (37). Further studies are thus warranted to elucidate the precise function of UmaA in species other than M. smegmatis.

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