Tracking the Putative Biosynthetic Precursors of Oxygenated Mycolates of Mycobacterium tuberculosis

STRUCTURAL ANALYSIS OF FATTY ACIDS OF A MUTANT STRAIN DEVOID OF METHOXY- AND KETOMYCOLATES

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Disruption of the mma4 gene (renamed hma) of Mycobacterium tuberculosis has yielded a mutant strain defective in the synthesis of both keto- and methoxymycolates, with an altered cell-wall permeabilty to small molecules and a decreased virulence in the mouse model of infection (Dubre, E., Chan, J., Raynaud, C., Mohan, V. P., Lanne, M. A., Yu, K., Quémard, A., Smith, I., and Daffé, M. (2000) Mol. Microbiol. 36, 630–637). Assuming that the mutant would accumulate the putative precursors of the oxygenated mycolates of M. tuberculosis, a detailed structural analysis of mycolates from the hma-inactivated strain was performed using a combination of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, proton NMR spectroscopy, and chemical degradation techniques. These consisted most exclusively of α-mycolates, composed of equal amounts of C77-C79 dicyclopropanated (α1) and of C77-C79 monoethylenic monocyclopropanated (α2) mycolates, the double bond being located at the “distal” position. In addition, small amounts of cis-epoxymycolates, structurally related to α2-mycolates, was produced by the mutant strain. Complementation of the hma-inactivated mutant with the wild-type gene resulted in the disappearance of the newly identified mycolates and the production of keto- and methoxymycolates of M. tuberculosis. Introduction of the hma gene in Mycobacterium smegmatis led to the lowering of diethylenic mycolates of the recipient strain and the production of keto- and methoxymycolates. These data indicate that long-chain ethylenic compounds may be the precursors of the oxygenated mycolates of M. tuberculosis. Because the lack of production of several methyltransferases involved in the biosynthesis of mycolates is known to decrease the virulence of the tubercle bacillus, the identification of the substrates of these enzymes should help in the design of inhibitors of the growth of M. tuberculosis.

Mycolic acids, α-branched β-hydroxylated long-chain fatty acids (up to 90 carbon atoms), are the hallmark of the Mycobacterium genus that comprises several human pathogens such as Mycobacterium tuberculosis and Mycobacterium leprae, the causative agents of tuberculosis and leprosy, respectively. These molecules represent major cell envelope components (40–60% of the cell dry weight) and are found covalently linked to the cell wall arabinogalactan or esterifying trehalose and glycerol; both types of mycolic acid-containing components are believed to play a crucial role in the structure and function of the mycobacterial cell envelope (1–3). Mycolic acids attached to the cell wall arabinogalactan are organized with other lipids to form an outer permeability barrier with an extremely low fluidity that confers an exceptional low permeability to mycobacteria and may explain their intrinsic resistance to many antibiotics (4). Trehalose mycolates have been implicated in numerous biological functions related both to the physiology and virulence of Mycobacterium tuberculosis (3).

Numerous studies have been and are currently devoted to the structures and biosynthesis of these acids, primarily because these substances are specific to the Mycobacterium genus, and their metabolism is the only clearly identified target inhibited by the major antitubercular drug, isoniazid (5–8). With the re-emergence of tuberculosis infections caused by multidrug-resistant strains and the need for the development of new tuberculous drugs, deciphering the biosynthesis pathway leading to mycolates still represents a major objective of researchers. However, it remains that, despite the intensive efforts of biochemists over decades (3, 9–10) and, more recently, the help of molecular genetic (11, 12), the biosynthesis pathway leading to mycolic acids is still poorly understood. Nevertheless, it is currently admitted that the two known mycobacterial fatty-acid synthases (FAS) participate in the formation of all types of mycolates or their precursors. FAS-I, a synthase that has been shown to be a bimodal system, is necessary to produce C16, 18 and C22–26 saturated fatty acids, which may be either directly incorporated in mycolates as the α-branched chain or used as substrates of the elongation system, FAS-II. The finding that isoniazid strongly and specifically inhibits InhA, an enoyl-acyl carrier protein reductase that belongs to FAS-II (13–14), is consistent with the proposed biosynthetic pathway leading to the various types of mycolates.

Mycolic acids occur usually in mycobacterial species as a mixture of various related molecules that differ from one an-

This paper is available online at http://www.jbc.org
other by the presence of chemical groups located on well-defined positions of their long methylene ("meromycolic") chain. In members of the *M. tuberculosis* complex, three types of mycolates are commonly encountered (15, 16). The least polar mycolates, also called */H9251*-mycolates, are composed of C76-C82 fatty acids (17) and contain two cis-cyclopropyl groups, at the so-called "proximal" and "distal" (relative to the carboxyl group) positions of the meromycolic chain (see Fig. 1 A). The more polar "M" and "K" mycolates consist of C82-C89 substances (17) and contain a cis-or a trans (with an adjacent methyl group)-cyclopropyl group at the proximal position, and a methoxy- or a keto- group (with an adjacent methyl group) at the distal position (Fig. 1 A). These discrete structural variations in mycolates may be of crucial biological importance, because it has been shown that mutations resulting in the loss of these chemical functions profoundly modify the permeability of the cell envelope to solutes and severely affect the virulence of the mutant strains in experimental infections (1, 18, 19). Accordingly, the enzymatic systems that introduce the chemical modifications in the mycolic acid chain merit special attention. Based on C-alkylation mechanisms (20), biosynthetic pathways that may explain the action of specific S-adenosylmethionine-dependent methyltransferases on ethylenic precursors leading to methyl branches and cyclopropanes in mycolates has been postulated (11). Similar C-alkylation mechanisms have been proposed to explain the synthesis of keto- and methoxy-mycolic acids: the transformation of the distal double bond of a precursor into a secondary hydroxyl group with an adjacent methyl branch using an S-adenosylmethionine-dependent methyltransferase coded by the gene *mma4* (21). A gene with the same function in *Mycobacterium bovis* BCG, first called *cmaA* (22), when introduced in *Mycobacterium smegmatis* has been shown to confer to the latter organism the ability to produce ketomycolic acids. In addition, the transformant produced large amounts of hydroxymycolic acids with an adjacent methyl-branch, structurally related to the ketomycolic acids of *M. bovis* BCG. Trace amounts of these hydroxymycolic acids have been also detected in mycobacterial species producing keto- and/or methoxymycolates, further supporting the hypothesis that hydroxymycolic acids can be the precursors of both keto- and methoxymycolic acids (23). Finally, a mutant strain of *M. tuberculosis* in which the *mma4* gene (thereafter called *hma* for hydroxymycolic acid) has been inactivated and shown to be devoid of both keto- and methoxymycolic acids (1, 17). Although this last experiment clearly established the biosynthetic relationships between the three oxygenated mycolates of *M. tuberculosis*, the chemical structures of precursor molecules used by Hma to produce these oxygenated mycolates remain unknown. Interestingly, analysis of the mass spectra of the fatty acids from the mutant strain indicated the accumulation of new substances structurally related to */H9251*-mycolates and absent from the parent strain (17). In an attempt to identify these

Fig. 1. Structures of the major mycolic acids of *M. tuberculosis* H37Rv (A) and *hma*::hyg mutant (B). The total carbon number refers to data obtained from the MALDI-TOF mass spectra. The mass values of the major homologues are represented in boldface. The values of *m*1 (the number of methylene groups in the distal part) are indicated; *m*2 and *m*3 depend on the presence or the absence of methyl branch adjacent to the double bond and cyclopropane on the meromycolic acid chains. *m*1 = 26, 28 (α, α-mycolates); 33-35 (M, methoxymycolates); 33, 35 (K, ketomycolates); 30, 32 (α3, dicyclopropanated mycolates); 29, 31, 33 (α2, monoethylenic mycolates); 29-33 (E, epoxymycolates). *t* indicates the trans conformation of the proximal cyclopropane that is always accompanied by a methyl branch, explaining the odd-numbered values of the total carbon number.
putative precursors of oxygenated mycolic acids of *M. tuberculosis*, a detailed analysis of the mycolic acid content of the hna knock-out mutant was performed using the combination of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, proton nuclear magnetic resonance (1H NMR) spectroscopy, and various chemical degradation techniques.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—The wild-type *M. tuberculosis* H37Rv (ATCC 27294) transformed with pMV261, a replicative vector, its isogenic mutant strain referred as hna::hyg, obtained by disruption of the hna gene and the complemented mutant strain named pJD, carrying the wild-type hna gene on the multicopy plasmid pMV261 (1) were grown at 37°C on synthetic Sauton medium as surface pellicles for a few weeks. Kanamycin-resistant transformants of *M. smegmatis* mc²155 strain were obtained after electroporation with the replicative plasmid pMV261 containing the wild-type hna gene cloned downstream of the hs660 promoter (1). The resultant transformants were grown to stationary phase in 5 ml of 7H9 medium with kanamycin (10 μg ml⁻¹) as described previously (22) because of the observed frequent loss of the plasmid in 100-ml cultures.

**Purification of Mycolic Acids**—Whole cells or bacterial residues obtained after lipid extraction with organic solvents (1) were saponified by a mixture of 40% KOH and methoxyethanol (1.7, v/v) at 110°C in a screw-capped tube. After acidification, fatty acids were extracted with diethyl ether and methylated with an ethereal solution of diazomethane (15). The mycolate patterns of the strains were determined by analytical thin-layer chromatography (TLC) on Silica Gel 60 (Macherey-Nagel) using either eluent A (petroleum ether/diethyl ether; 9:1, v/v) or eluent B (dichloromethane). Revelation of lipid spots was performed by spraying the plates with molybdophosphoric acid (10% in ethanol), followed by charring. The crude mycolate fraction was obtained by precipitating an ethereal solution of fatty acid methyl esters with methanol at 4°C, followed by centrifugation at 4000 x g for 20 min (24). The different classes of mycolates were separated by chromatographic cleavage on Florisil columns 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 (17).

To search for the presence of ethylene compounds, the various purified mycolate types were analyzed and fractionated on AgNO₃-impregnated silica gel TLC plates developed with eluent B (CH₃Cl) or eluent C (petroleum ether/diethyl ether; 9:1, v/v). The various purified types and sub-types of mycolates were quantified by weighing and radiolabeling with [¹⁴C]acetate.

**Radiolabeling**—30 μl of [¹⁴C]acetic acid sodium salt (2 MBq/mlmol, Amersham Biosciences) were added to a 30 ml of surface cultures of the H37Rv and hna::hyg strains of *M. tuberculosis*, and the incubation was left at 37°C. The culture media were discarded, and the cell pellets were saponified as described above; the resulting fatty acids were methylated and analyzed by both silica gel-coated and argentated (AgNO₃-impregnated) TLC using dichloromethane as eluent. The radiolabeling was determined using a PhosphorImager (Amersham Biosciences).

**Degradative Techniques**—Cleavage of double bonds was performed by permanganate-periodate oxidation (25) at 30°C in tertiary butanol as solvent. Acetylation of epoxymycolates was realized by adding acetic acid to the purified mycolates at reflux for 40 h, followed by saponification to liberate the corresponding diols. The resulting compounds were submitted to oxidative cleavage as above. The acids obtained from the diolomycetes 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 CDCl₃ (100% D) using a Bruker AMX-500 spectrometer at 298 K. Chemical shifts values (in ppm) were relative to the internal CHCl₃ resonance (at 7.27 ppm). MALDI-TOF mass spectra (in the positive mode) were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser emitting at 337 nm. Samples were analyzed in the Reflection mode using an extraction delay time set at 100 ns and an accelerating voltage operating in positive ion mode of 20 kV. To improve the signal-to-noise ratio, 150 scans were averaged for each mass spectrum, and typically, four individual spectra were accumulated to generate a summed spectrum. Mass spectrum calibration was performed using the calibration mixture 1 of the Sequazyme empowered peptide mass standards kit (PerSeptive Biosystems), including known peptide standards in a mass range from 900 to 1600 Da. Internal mass calibration was performed with sodiated synthetic corymycolic acid ([C₃₀H₆₀O₃ + Na]⁺, M, 519.6748) and its methyl ester derivative ([C₃₀H₆₁O₃ + Na]⁺, M, 533.4904) or with the sodiated C₇H₁₄O₄ mycolic acid methyl ester previously described ([M + Na]⁺ = 1146.1477). The stock solutions of mycolates were prepared in chloroform, at a concentration of 1 mM, and were directly applied on the sample plate as 1-μl droplets, followed by the addition of 0.5 μl of matrix solution (2.5-dihydroxybenzoic acid (10 mg/ml) in CHCl₃/CH₃OH (1:1, v/v)). Samples were allowed to crystallize at room temperature.

Infrared spectra of samples as films on NaCl discs were recorded using a PerkinElmer Life Sciences Fourier transform IR 1600 spectrometer.

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 x 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. GC-mass spectrometry (GC-MS) analyses were performed on an HP 5889X mass spectrometer (electron energy, 70 eV) coupled to an HP 5890 series II gas chromatogram fitted with a column identical to that used for GC. GC-MS analyses were realized in the electron impact mode.

**RESULTS**

**Lipid Associated With the Disruption of the hna Gene**—The parent strain (H37Rv) of *M. tuberculosis* and its isogenic hna-disrupted mutant exhibited similar growth rates both on synthetic media and during the infection of the macrophage-like THP-1 cell line (1). They were similar both in their content in extractable lipids (20–21% of the bacterial dry weights) and in wall-linked mycolates (10%). No obvious difference was seen between the two strains in terms of major extractable lipids, including sulfatides, triacylglycerol, glycerol mycolate, trehalose monomycolate, trehalose dimycolates, and phospholipids (data not shown). They also showed identical C₅₀C₀₂₀ fatty acid methyl ester profiles. They differed, however, in the types of mycolic acids esterifying trehalose and glycol in extractable lipids, and those linked to the cell wall arabinogalactan. Although the parent strain contained the three characteristic types of mycolates, namely α-, methoxy-, and ketomycolates, the mutant contained most exclusively α-mycolates (Fig. 2A). Because the two strains contained similar amounts of mycolic acids, the mutant strain produced more α-mycolates than the parent strain to compensate for the absence of oxygenated mycolates. Nevertheless, the lack of production of oxygenated mycolates resulted in a profound alteration of the cell envelope permeability (1). That this phenotype was due to the inactivation of the *hna* gene was shown by the complementation of the mutant strain with the wild-type *hna* gene. Analysis of the mycolate profile of the complemented strain showed that the production of both keto- and methoxymycolates was restored (Fig. 2A), but the ratio between the two oxygenated mycolates differed from that of the parent strain. This difference, attributed to the overexpression of the *hna* gene in the mutant, was found to affect the permeability of cell wall barrier of the complemented strain to phenolmycolate. The complemented strain exhibited a lower initial rate of uptake and less accumulation of the probe compared with the parent strain but higher than that of the *hna*-inactivated mutant (data not shown).

Examination of the MALDI-TOF mass spectrum of the whole cell fatty acid methyl esters of the complemented strain confirmed the above data. The mass spectrum from the strain complemented with the wild-type *hna* gene on a multicopy plasmid contained the two massifs of peaks previously observed in the spectrum of the parent strain (17), i.e. a massif assignable to α-mycolates (at m/z 1146 to 1202) and another attributable to oxygenated mycolates (at m/z 1218 to 1330). This latter massif was absent from the mass spectrum of fatty.
Acid methyl esters from the hma-inactivated mutant (17). In agreement with the TLC data (Fig. 2A), the content in peaks corresponding to methoxymycolic acids (pseudomolecular ion peaks at \( m/z \) 1262 for C85 and at \( m/z \) 1290 for C86) was very low in the mass spectrum of the complemented strain, compared with that of ketomycolic acids (pseudomolecular ions at \( m/z \) 1246 for C82, 1274 for C84, and 1302 for C86). Pseudomolecular ion peaks, at \( m/z \) 1288 (C85) and \( m/z \) 1316 (C87) were attributed to the presence of small amounts of ketomycolic acids with odd-carbon number, commonly occurring in ketomycolates with one α-branch trans cyclopropane (17). It follows then that the production of oxygenated mycolates by the complemented strain was due to the presence of the hma gene.

**Definition of the Types of Mycolic Acids in the hma::hyg Mutant**—The MALDI-TOF mass spectrum of the fatty acid methyl esters from the hma-inactivated mutant contained only one massif of peaks, in the region between \( m/z \) 1118 and 1272, corresponding to α-mycolic acids, but was much more complex than that of the wild-type. Although the massif of α-mycolic acids from the parent strain consisted of four major pseudomolecular [M + Na]⁺ ions corresponding to α-mycolic acids composed of C76, C78, C80, and C82, that of the mutant strain contained at least 10 major ion peaks that differed from one another by 14 atomic mass units, indicating the existence of mycolic acids with odd and even carbon numbers (C76 to C82). To analyze further the mycolic acids of the hma mutant, the mycolate-enriched methanol precipitate was first examined by TLC (Fig. 2A). This analysis confirmed the predominance of α-mycolic acid methyl esters and showed the presence of an additional polar compound with a mobility lower than that of ketomycolic acid methyl esters. The methanol precipitate was then fractionated by chromatography on a Florisil column. The least polar fraction, consisting exclusively of α-mycolic acid methyl esters, was analyzed by ¹H NMR spectroscopy comparatively to α-mycolates from the parent and complemented strains (Fig. 3). The three spectra of α-mycolates contained the expected signals attributed to cyclopropanated mycolates. The isolated methylene proton resonances (at 1.29 ppm, broad signal), signals due to terminal methyl groups (at 0.85 ppm, triplet), methyl ester (at 3.71 ppm, singlet), methine (at 4.70 ppm, broad signal), signals due to terminal methyl groups (at 0.85 ppm, triplet), methyl ester (at 3.71 ppm, singlet), and signals assignable to cis-cyclopropyl proton resonances (at 0.35, 0.45, and 0.70 ppm) were clearly identified. In addition, the ¹H NMR spectrum of the α-mycolates from the hma mutant contained signals attributable to ethylenic proton resonances (at 5.34 ppm) and those of methylene adjacent to double bonds (at 2.00 ppm). These latter signals were absent from the spectra of α-mycolates isolated from the parent and complemented strains. Thus, the analysis of the ¹H NMR spectra confirmed the structural difference observed by MALDI-TOF mass spectrometry between the α-mycolates from the hma mutant and those of the parent strain and showed the occurrence of double bonds in the mycolates produced by the mutant strain. Interestingly, the ¹H NMR spectra of the α-mycolates from the parent (Fig. 3) and complemented strains (data not shown) were superimposable, indicating that the production of ethylenic compounds by the mutant strain was due to the inactivation of the hma gene.

To determine the origin of the structural difference between the α-mycolates from the mutant strain and those of the parent and complemented strains, the α-mycolates from the mutant were further analyzed by AgNO₃-impregnated TLC (Fig. 2B), a layer that is known to have an affinity for cis-ethylenic bonds.

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**Fig. 2.** Thin-layer chromatography of the methyl esters of mycolic acids from M. tuberculosis. A: silica gel as adsorbent; I, H₃Rv (pMV261); 2, hma::hyg mutant; 3, hma::hyg mutant complemented with the wild-type hma gene; 4, hma::hyg mutant (methanol-insoluble esters); α, α-mycolates; M, methoxymycolates; K, ketomycolates; E, epoxymycolates. Solvent: petroleum ether/diethyl ether 9:1 (v/v, five runs). B: argentation chromatography of α-mycolates from: I, H₃Rv (plasmid pMV261); 2, hma::hyg mutant; 3, hma::hyg mutant complemented with the wild-type hma gene (pJD). α₁, α₂, and α₃ refer to dicyclopropanated, monomethylenic, and monocyclopropanated, and diethylenic-α mycolates, respectively. Solvent: dichloroethane. Visualization for A and B: molybophosphoric acid followed by charring. Arrows indicate the solvent front.

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**Table 1**

Comparative data from MALDI-TOF mass spectrometry of mycolic acids methyl esters from M. tuberculosis H₃Rv (parent strain), hma (hma::hyg mutant), and pJD (hma::hyg complemented with the wild-type hma gene).

| Mycolic acid type | Total carbon number of free acid | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 |
|------------------|---------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| α Rv             |                                 | 1146| 1174| 1202| 1230|
| α pJD            |                                 | 1146| 1174| 1202| 1230|
| α hma            |                                 | 1118| 1132| 1146| 1160| 1174| 1188| 1202| 1216| 1230|
| Methoxy Rv       |                                 | 1262| 1290| 1318| 1346|
| Methoxy pJD      |                                 | 1262| 1290| 1318| 1346|
| Keto Rv          |                                 | 1246| 1274| 1288| 1302| 1316| 1330|
| Keto pJD         |                                 | 1246| 1274| 1288| 1302| 1316| 1330|

Values represent the pseudomolecular masses of mycolic acids methyl esters [M + Na]⁺. The mass values of the major homologues are represented in boldface.
Two major lipid spots, called $\alpha_1$ and $\alpha_2$, were detected and isolated by preparative AgNO$_3$-impregnated TLC and represented 90–95% (by weight) of the $\alpha$/H$_9251$-mycolic acid methyl esters from the mutant strain. A lipid spot with a lower mobility, called $\alpha_3$ (Fig. 2B), was also detected. The spots corresponding to $\alpha$/H$_9251$-2- and $\alpha$/H$_9251$-3-mycolates were not detected in mycolates either of the parental strain or the $hma$-complemented strain (Fig. 2B). The $\alpha$/H$_9251$-1-mycolates from the mutant strain exhibited the same mobility on AgNO$_3$-impregnated TLC as the dicyclopropanated $\alpha$/H$_9251$-mycolates from the parent strain, whereas the $\alpha$/H$_9251$-2- and $\alpha$/H$_9251$-3-mycolates had a migration consistent with the presence of one and two cis-ethylenic double bonds, respectively. Quantification experiments indicated that $\alpha$/H$_9251$-1-mycolates represented 45% (by weight) of the mycolates isolated from the mutant strain $hma$ and consisted of peaks corresponding to C$_{74}$-C$_{84}$ $\alpha$/H$_9251$-mycolates (Fig. 5 and Table II). Major peaks in the spectrum of $\alpha$/H$_9251$-2-mycolates from the $hma$-inactivated strain corresponded to the odd-numbered carbon atoms (C$_{75}$ to C$_{83}$), consistent with the $\alpha$/H NMR data that indicated the presence of one double bond and one cyclopropyl in these molecules. The mass spectrum of $\alpha$/H$_9251$-3-mycolates was more complex. The major series of peaks corresponded to compounds possessing C$_{76}$ to C$_{80}$ and two cis-double bonds (Fig. 5 and Table II). Two minor series of peaks were also observed and could tentatively be attributed to mycolates having either (i) three cis-double bonds, (ii) two cis-double bonds and a cyclopropyl, or (iii) two cis-double bonds and a trans-double bond with an adjacent methyl branch. The postulated cyclopropyl and trans-double bond were not observed in the $\alpha$/H NMR spectrum of $\alpha$/H$_9251$-mycolates (Fig. 4), probably due to their relative small abundance in the mixture. Interestingly, minor amounts of $\alpha$-mycolates possessing three unsaturation centers have been reported to occur in mycobacterial strains (26).
Additional data on the location of the various chemical functions in mycolates were obtained by mass spectrometry in the electron impact (EI) mode, notably by the occurrence of ion peaks due to rearrangements of the aldehyde fragments derived from the pyrolysis of the molecules (27–30). Indeed, as expected, a prominent peak at 410 m/z was observed in all the EI mass spectra of the mycolates examined and corresponded to the hexacosanoic acid methyl ester released from the pyrolytic cleavage of mycolates from members of the \textit{M. tuberculosis} complex (15). Furthermore, in the EI mass spectra of the dicyclopropanated \textit{H9251}-mycolates from the parent and complemented strains and \textit{H9251}-1-mycolates from the \textit{hma}-inactivated mutant strain, peaks were observed and corresponded to the "meroaldehydes" with 52, 54, and 56 carbon atoms, respectively, at 740, 768, and 796 m/z. However, differences were seen between the EI mass spectra of \textit{H9251}- and \textit{H9251}-1-mycolates in the relative abundances of peaks that allowed the determination of $m_1$ values, i.e. the number of methylene groups in the distal part of the molecule (Fig. 1). These resulted from the interactions between the meroaldehydes and the distal cyclopropane (27, 28). Although the major fragmentation peaks of the meroaldehydes were observed at 459 and 487 m/z in the spectrum of \textit{H9251}-mycolates from the parent strain, the corresponding peaks in the spectrum of the \textit{H9251}-mycolates from the \textit{hma}-inactivated mutant strain were seen at m/z 459 and 487 but also at higher mass values (m/z 515 and 543). These data indicated that, although the mutant strain elaborated \textit{H9251}-mycolates with chain lengths identical to those of the \textit{H}-mycolates of the parent strain, the two strains differed one another by the $m_1$ values of dicyclopropanated mycolates (Fig. 1). A more complex pattern of peaks was seen in the region of meroaldehydes of the spectrum of \textit{H9251}-mycolates isolated from the \textit{hma}-inactivated mutant strain. This was probably due to the occurrence of a double bond that stabilizes the secondary fragmentation ions. To circumvent this difficulty and localize the double bond in \textit{H9251}-mycolates, the molecules were first submitted to an oxidative cleavage (25), a method that cleaves the compounds at the level of the ethylenic bond to yield mono- and dicarboxylic acids. When the resulting fatty acids were esterified, purified, and analyzed by GC and GC-MS, heptadecanoic and nonadecanoic acid methyl esters were identified as the monoesters, establishing the values of $m_1$ (Fig. 1). Similarly, the purified long chain diester cleavage products were analyzed by MALDI-TOF mass spectrometry, comparatively to the \textit{H}-carboxylic acid methyl ester of similar structure isolated from \textit{Mycobacterium phlei} (17). The MALDI-TOF mass spectrum of the long chain diacid methyl esters gave peaks at m/z values 968, 996, and 1024, consistent with the occurrence of molecules with chain lengths of C$_{60}$, C$_{62}$, and C$_{64}$ for the free diacids. Thus, the data on the cleavage products showed that a cyclopropyl group, insensitive to oxidative cleavage, was located at the proximal position of the \textit{H9251}-mycolates and confirmed that intact \textit{H9251}-mycolic acids contained the odd-
numbered carbon atoms as deduced from the analysis of their intact MALDI-TOF mass spectra.

Structure of Oxygenated Mycolates of hma::hyg Strain—Compound E produced by the mutant in small amounts exhibiting the lowest Rs (Fig. 2A) was purified by chromatography on a Florisil column. It represented 5% (by weight) of the mycolic acid methyl esters of the mutant and was analyzed by various analytical techniques. The occurrence of mycolic structure, i.e. \( \alpha \)-branched \( \beta \)-hydroxylated fatty acid, in the compound was confirmed by the detection of hexacosanoic acid methyl ester released upon a pyrolytic cleavage on GC. Analysis of the IR spectrum of compound E revealed the presence of an infrared absorption band at 850 cm\(^{-1}\) corresponding to the absorption band of a cis-epoxy group, that of a trans-epoxy ring in mycolates being observed at 900 cm\(^{-1}\) (31). These data were confirmed by NMR spectroscopy in that the \(^1\)H NMR spectrum of compound E from the hma-inactivated strain of \( M. \) tuberculosis showed a signal at 2.90 ppm (Fig. 3), whereas the characteristic resonance of the trans-epoxymycolic acid methyl esters (bearing a methyl branch adjacent to the epoxide) from \( M. \) fortuitum \( fortuitum \) was seen at 2.71 ppm (31–33). The observed chemical shift values were consistent with those reported for synthetic epoxy acids (34) in which the resonances of cis- and trans-epoxide were seen at 2.90 and 2.65 ppm, respectively. The cis configuration of the epoxy ring in compound E from the hma-disrupted mutant strain was also supported by the absence of the characteristic doublet at 1.10 ppm observed in the epoxymycolates from \( M. \) fortuitum and \( M. \) smegmatis (31–33) and assigned to the methyl branch adjacent to the epoxy ring. The \(^1\)H NMR spectrum of compound E also showed the presence of the three characteristic signals of a cis-cyclopropyl ring (Fig. 3). To characterize compound E further, the purified mycolate was analyzed by MALDI-TOF mass spectrometry (Table II). Pseudomolecular ion peaks were seen at 1176, 1204, 1232, 1260, and 1288 m/z, corresponding to free acids with 77 to 85 carbon atoms. Analysis of the EI mass spectrum confirmed the molecular mass determination of compound E in that the constitutive mycolic acid methyl esters gave the expected ion peaks corresponding to pyrolytic cleavage products: an ion peak at 410 m/z attributed to the released hexacosanoic acid methyl ester and a series of peaks of “anhydromeroaldehydes” having 51, 53, and 55 carbon atoms at 724, 752, and 780 m/z. The values of \( m_1 \) in compound E were determined by oxidative cleavage of the epoxide, which was first transformed into an \( \alpha \)-dil and then cleaved by peridate to yield long chain fatty acids and dicarboxylic acids. The resulting reaction products were methylated, purified, and analyzed by GC-MS and MALDI-TOF mass spectrometry (data not shown). The data allowed the location of the epoxy group in compound E at the same position as the distal ethylenic bond in the \( \alpha_2 \)-mycolates (Fig. 1B).

Minor signals assignable to double bonds (at 5.34 ppm) were also observed in the \(^1\)H NMR spectrum of compound E (Fig. 3). Accordingly, the suspected heterogeneity of the mycolate was resolved by argentation chromatography. The major constituent of compound E, called epoxy-2, exhibited a mobility higher than that of the purified epoxymycolates of \( M. \) fortuitum, which contain a double bond instead of a cyclopropyl group, as expected. In addition, a minor polar lipid spot, with a mobility similar to that of the epoxymycolates of \( M. \) fortuitum, was present in compound E. The two classes of epoxymycolates from compound E were purified by preparative argentation chromatography and analyzed by MALDI-TOF mass spectrometry (Table II). Epoxy-2, the least polar compound, corresponded to cyclopropanated cis-epoxymycolates having 77–85 carbon atoms, whereas the minor and most polar constituent, epoxy-3, consisted mainly of epoxymycolates containing one double bond; more unsaturated compounds (two double bonds

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**Table II**

*Data from MALDI-TOF mass spectrometry of the methyl mycolic acids methyl esters from the hma mutant strain*

| Total carbon number of free acid | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 |
|---------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| \( \alpha_1 \)                  | 1118| 1146| 1174|    |    |    | 1202| 1230|    |    |    |    |    |    |    |
| \( \alpha_2 \)                  | 1090| 1118| 1146| 1174|    |    | 1188| 1202| 1230|    |    |    |    |    |    |
| **Epoxy 2**                     |    |    |    |    |    |    |    |    |    | 1204| 1232| 1260|    | 1288|    |
| **Epoxy 3**                     |    | 1162| 1176| 1190| 1204| 1218| 1246| 1260| 1274| 1288|    |    |    |    |    |

*CP*, cyclopropane; *DB*, double bond.
or one double bond and a cyclopropyl ring) were also present in epoxy-3, as observed for α-mycolates from the mutant strain (see above and Table II). It was thus concluded that compound E consists mainly of cyclopropanated cis-epoxymycolates and contained minor ethylenic constituents with two degrees of unsaturations showing a structural relationship with α-mycolates of the mutant strain.

Kinetics of Production of the Different Types of Mycolates Synthesized by the hma::hyg Mutant Strain of M. tuberculosis—To determine whether the α- and epoxymycolates that are accumulated in the hma-inactivated mutant arise as a consequence of secondary reactions catalyzed by other enzymes, cells from the parent and mutant strains of M. tuberculosis were labeled at different time points of their growth phases by [1-14C]acetate, and the resulting mycolates were purified and analyzed by radio-TLC. Cells from both log phase (6 days old) and stationary phase (23 days old) bacteria, produced roughly equal amounts of the dicyclopropanated (cis) and monoethylenic (αα) mycolates (Fig. 6). Although the very minor epoxymycolates were poorly labeled (roughly 4% of mycolate labeling), as expected, they were seen in all the growth stages of the mutant strain. It was thus unlikely that the new α-mycolates, which are produced in the hma-inactivated mutant, arise from secondary reactions. This conclusion was further supported by the results of pulse-chase experiments (data not shown) in which young cells were labeled with [1-14C]acetate for 24 h and then transferred in fresh sterile media for 2–33 days. No significant change was observed in the kinetics of incorporation and amounts of incorporated radioactivity into the various classes of lipids.

Overexpression of hma in M. smegmatis and MALDI-TOF Mass Spectrometry Analysis of Mycolates from the Transformed Strain—The mycolate pattern of M. smegmatis is different from that of M. tuberculosis, especially because the former mycobacterial species does not contain methoxy- and ketomycolates (22). Instead, M. smegmatis elaborates C75–C84 epoxy-mycolic acids, short-chain monounsaturated C60–66 mycolic acids (called α′), and diunsaturated C75–C84 α-mycolic acids. Interestingly, the α-mycolates of M. smegmatis are structurally similar to the αα-mycolates of the hma::hyg mutant strain of M. tuberculosis (17, 22). Therefore, M. smegmatis was used as a worthwhile host to address further the question of the possible biosynthetic relationship between the ethylenic mycolates that accumulated in the hma::hyg mutant strain and the oxygenated mycolates whose production was abolished in the mutant. Accordingly, the hma gene from M. tuberculosis was cloned and overexpressed in M. smegmatis, and the resulting transformant clones were screened by TLC analysis for the presence of keto- and hydroxymycolates (22). Interestingly, both TLC (data not shown) and MALDI-TOF mass spectrometry analyses (Fig. 7) showed that the amounts of α-mycolates was severely reduced, compared with the parent strain carrying the vector alone, in all the clones producing keto- and hydroxymycolates, in addition to the usual mycolic acids of M. smegmatis. Analysis of the MALDI-TOF mass spectra showed that the parent strain of M. smegmatis produced mainly C77 and C79 α-mycolic acids (Fig. 7A) and the overexpression of the M. tuberculosis hma gene in M. smegmatis strain led to the synthesis of mainly C78 and C80 keto- and hydroxymycolates (Fig. 7B). These data are consistent with the hypothesis that α-mycolic acids or precursor molecules are used to produce the keto- and related hydroxymycolates in mycobacterial strains expressing the hma gene.

DISCUSSION

We have previously shown that the introduction of the gene cmaA from M. bovis BCG Pasteur into M. smegmatis induced the production by the recipient strain of large amounts of methyl-branched hydroxymycolic acids and small amounts of ketomycolic acids (22). Inactivation of the corresponding gene
of *M. tuberculosis* H37Rv, *hma* (or *mma4*), has resulted in the complete abolishment of the production of all the oxygenated mycolates normally present in *M. tuberculosis* H37Rv, namely the methoxy-, keto-, and hydroxymycolic acids (1). It was thus concluded that the *hma* gene is involved in the synthesis of oxygenated mycolic acids and that hydroxymycolic acids are likely to be precursors of methoxy- and ketomycolates in *M. tuberculosis*. Interestingly, the *hma*-disrupted mutant strain was found to produce new types of mycolic acids absent from the parent strain, suggesting that these molecules may represent the precursors of the oxygenated mycolates of *M. tuberculosis* (17). Accordingly, the present work was undertaken to elucidate the chemical structures of these molecules. 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 mutant established several facts. First, in the absence of the *hma* gene, the mutant strain synthesized as much mycolates as the parent strain, suggesting that the overall bacterial content in these molecules is important for the normal physiology of the bacilli. Second, both the mutant and parent strains synthesized similar amounts of dicyclopropanated α-mycolates exhibiting identical structures, indicating that the mutation did not affect the production of this type of mycolates. Third, the mutant produced large amounts of a new type of mycolates, namely a mixture of monoethylenic monocyclopropanated α-mycolates, and a tiny amount of cis-epoxy-containing monocyclopropanated mycolates; the ethylenic α-mycolates were synthesized throughout the various growth phases of the mutant, indicating that they did not arise from secondary reactions. Fourth, complementation of the mutant strain with the wild-type *hma* gene resulted in the disappearance of the new types of mycolates and the production by the complemented strain of all the types of mycolates of the parent strain. Finally, introduction of the *hma* gene in *M. smegmatis* led to the lowering of diethylenic α-mycolates of the recipient strain and the production of keto-and hydroxymycolates. Our results demonstrate that the observed changes in the structure of mycolates of the mutant are specifically due to the inactivation of the *hma* gene and raise some important questions regarding the biosynthesis of mycolic acids.

One of the questions related to the biosynthesis of mycolic acids is the metabolic process that leads to the introduction of the specific oxygenated functions at the distal position of the molecules. Two classes of substrates could lead to oxygenated chemical functions after a C-methylation (20, 35), namely a keto group, leading to an α-methylated ketone (Fig. 5A), as known for menaquinones, or an ethylenic group (Fig. 5B); in this latter case a water molecule should participate in the sulphonium-mediated addition mechanism, as proposed for Mma4 function (21). Because only cis-ethylenic mycolate accumulates significantly in the *hma*-inactivated mutant, it is more attractive to postulate that the ethylenic distal double bond occurring in the new α-mycolates of the mutant strain is the substrate of Mma/Hma. In the "ethylenic hypothesis" (Fig. 8B), the hydroxyl group formed after the methylation step of the ethylenic bond could be either transformed into a methoxy group or oxidized into a ketone. In both cases, an oxidoreduction step would be necessary to obtain either the keto- or the methoxymycolic acids. Importantly, the values of m1 in the new α-mycolates are identical to those found in oxygenated mycolates of the parent strain and shorter than those observed in the α-mycolates of the parent strain (Fig. 1). Interestingly, the difference in chain lengths between α-mycolates and oxygenated mycolates have been previously observed (17, 36) and shown to be due to the specificity of the methyltransferases mainly with respect to the α-end of the growing mero acyl chain (37).

![Fig. 8. Possible reaction mechanisms for the synthesis of oxygenated mycolic acids mediated by the Hma methyltransferase (adapted from Ref. 20). A, the "ketone hypothesis"; B, the "ethylenic hypothesis."](image_url)

Based on the kinetics of production of α-mycolates and related oxygenated compounds in *Mycobacterium microti* (38) and other mycobacterial species (39) on the one hand, and the chain lengths of these two classes of substances, on the other hand, it has been suggested that these molecules are synthesized by different enzymatic systems. Accordingly, the common precursor, if any, would not be a full "meromycolic" diethylenic compound that would then be modified by different methyltransferases, before or after the Claisen-type condensation step, to yield α-or/and oxygenated-mycolic acids (for a review see Ref. 3). By showing that the *hma*-disrupted mutant produced dicyclopropanated α-mycolic acids as the parent strain and in comparable amounts, our data are consistent with the existence of one biosynthetic system devoted to the synthesis of α-mycolic acids and another machinery involved in the synthesis of oxygenated mycolic acids; only the latter one would be altered by the disruption of the *hma* gene. Both systems may contain a mixture of "core" enzymes whose activities are necessary for the synthesis of the meromycolic chain but may differ one from another by the association of additional specific enzymes involved in the introduction of various chemical groups in the meromycolic chain. The accumulation of large amounts of ethylenic α-mycolates in the *hma*-inactivated mutant, comparable to those of oxygenated mycolates of the parent strain, reinforces the hypothesis that ethylenic long chain fatty acid derivatives may be used for the biosynthesis of oxygenated mycolates in the parent and the *hma*-complemented strain. In the absence of the *hma* gene, these putative precursors would be used by the machinery originally devoted to the synthesis of oxygenated mycolates and yield distal-ethylenic compounds. The remaining unsolved question is the discrepancy between the chain lengths of the postulated precursors and those of the final products. Although the mutant produced C70–C84 monoethylenic monocyclopropanated α-mycolates (α2) and cis-epoxy monocyclopropanated mycolates, the parent and complemented strains synthesized C81–C90 methoxy- and ketomycolates, 4–6 carbon atoms longer than the α-mycolates (17, 26). Examination of the detailed structures of mycolic acids, however, pointed to the observation that the additional carbons in oxygenated mycolates are not located between the methyl end and the oxygenated group but distributed in the other parts of the meromycolic chain. Accordingly, one can postulate that the introduction of an oxygenated function at this position induces enough hydrophilicity in the long carbon chain to slightly disturb the specificity toward chain lengths of the system in charge of the synthesis of the methylenic chain.
As a consequence, the oxygenated groups should be introduced before completion of the meromycolic chain.

Several genes coding for methyltransferases have been identified in the genome of *M. tuberculosis* (40). These enzymes are assumed to be responsible for the introduction of subtle variations in the mycolic acid structure, variations that may have profound effects on the physiology and virulence of the tubercle bacillus; for instance, the replacement of a cyclopropane ring by a double bond in α-mycolates esterifying trehalose totally abolishes the formation of cords typifying virulent tubercle bacilli and profoundly affects the virulence of the mutant strain (19).

Similarly, the lack of production of keto- and methoxymycolates in *M. tuberculosis* results in both a change of the permeability and an attenuation of virulence of the mutant strain (1). These observations, in addition to the fact that the biosynthesis ability and an attenuation of virulence of the mutant strain (1). These observations, in addition to the fact that the biosynthesis ability and an attenuation of virulence of the mutant strain (1).

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