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The Specificity of Methyl Transferases Involved in *trans* Mycolic Acid Biosynthesis in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*

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Trans mycolic acid content is directly related to cell wall fluidity and permeability in mycobacteria. Carbon-13 NMR spectroscopy of mycolic acids isolated from *Mycobacterium tuberculosis* (MTB) and *Mycobacterium smegmatis* (MSM) fed ^{13}C-labeled precursor molecules was used to probe the biosynthetic pathways that modify mycolic acids. Heteronuclear correlation spectroscopy (HMQC) of ketomycolic acid from MTB allowed assignment of the complete ^{13}C-NMR spectrum. Incorporation patterns from [1-^{13}C]-acetate and [2-^{13}C]-acetate feeding experiments suggested that the mero chain and alpha branch of mycolic acids are both synthesized by standard fatty acid biosynthetic reactions. [^{13}C-*methyl*]-L-methionine was used to specifically label carbon atoms derived from the action of the methyl transferases involved in meromycolate modification. To enrich for *trans* mycolic acids a strain of MTB overexpressing the *mma1* gene was labeled. Carbon-carbon coupling was observed in mycolate samples doubly labeled with ^{13}C-acetate and [^{13}C-*methyl*]-L-methionine and this information was used to assess positional specificity of methyl transfer. In MTB such methyl groups were found to occur exclusively on carbons derived from the 2 position of acetate, while in MSM they occurred only on carbons derived from the 1 position. These results suggest that the MSM methyltransferase MMAS-1 operates in an inverted manner to that of MTB.

The cell wall of *Mycobacterium tuberculosis* (MTB) contributes directly to the success of this organism as an important pathogen of man by rendering it all but impervious to normal host defenses. The cell wall may in fact be the single most important virulence factor produced by the bacillus and the proper composition and assembly of this macromolecular complex has been directly linked to successful replication within host cells in several recent studies (*1–3*). The cell walls of species within the genus Mycobacteria rely upon covalently associated mycolic acids to form a protective pseudo outer membrane that is the major cell permeability barrier (*4–6*). Mycolic acids are extremely long-chain fatty acids ranging from 60–90 total carbons in the Mycobacteria. They have a common α-alkyl, β-hydroxy core structure with

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two long, lipophilic alkyl chains, the longer of which is typically functionalized in a species-specific fashion (see Fig. 1).

Although we understand the rudimentary principals of the structure of the cell wall complex, many of the details remain obscure (*7,8*). The mycobacterial cell wall, like other bacterial membrane systems, has a variable composition allowing the organism to maintain a constant viscosity in the face of changing environmental conditions (*9,10*). In typical bacterial membrane systems this is accomplished by adjusting both the length of the lipids produced as well as the ratio of unsaturated to saturated fatty acids produced. Among unsaturated fatty acids the ratio of *cis* to *trans* geometry further affects fluidity since the *trans* fatty acids, which pack more tightly, increase viscosity and thus lower permeability (*11*). Various mycobacterial species (including *M. avium* (*12*) and *M. smegmatis* (MSM) (*13,14*)) have also been shown to respond to changing growth temperature by adjusting the proportion of lipids found in the *trans* configuration. These alterations in lipid structure are not to plasma membrane lipids but rather to mycolic acids, as would be expected since the cell wall represents the major permeability barrier. In rapidly growing species, such as MSM, this alteration results in changes in the geometry of the major olefinic mycolic acids while in slowly growing species such as *M. avium* this alteration occurs through changes in geometry about cyclopropane rings within the longer meromycolate chain. This alteration has been shown to be directly correlated in these mycobacterial species with changes in cell wall permeability to lipophilic agents (*12*). In addition, patient isolates of MTB have been shown to synthesize different quantities of *trans* cyclopropanated mycolic acids than laboratory isolates, supporting a potential link between such alterations and continued growth *in vitro* (*15*).

Mycolic acids are biosynthesized through the same basic anabolic reactions used to construct all cellular lipids (*4*). Structural variety within these molecules results from the action of a family of methyltransferase enzymes that act upon olefinic precursors to produce the wide variety of chemical moieties observed. Among these modification reactions a number of enzymes involved in *cis* cyclopropane synthesis have been identified, including enzymes with apparent preference for modification of both the proximal and distal positions of the mycolic acid (*4*). These enzymes share significant sequence homology and as many as 8 discrete homologs of mycolic acid methyltransferases have been identified in the genome of MTB (*16*). These enzymes and their functions were identified by heterologous expression in the non-pathogenic saprophytic mycobacterium MSM. Using this species as a source of methyl group acceptor has also allowed us more recently to demonstrate the cell-free activities of several of these enzymes (*17*).

Despite these successes with heterologous expression of the *cis* cyclopropane synthases, similar strategies have been unsuccessful in obtaining the corresponding *trans* cyclopropane synthase gene(s) or protein(s). Using gene deletion studies in *M. tuberculosis* Glickman and coworkers recently demonstrated convincingly that one of the genes previously assigned as a *cis* synthase was, in fact, a *trans* synthase since the deletion mutant no longer produced *trans* cyclopropane-containing mycolic acids (*18*). The failure to observe the *trans* synthase activity upon heterologous expression was surprising because of the apparent prevalence of what appeared to be the correct precursor molecule, namely the alpha-methyl branched, *trans* olefinic $\alpha 2$ mycolic

acid of MSM (See Fig. 2). An examination of the fine structures of the MTB mycolic acids as they were originally reported by Minnikin and Polgar suggested a possible reason for this failure (*19*,*20*). In these structures the methyl branch was proposed to lie on the side of the cyclopropane closer to the ω end of the meromycolate chain based upon the pattern of ions observed in the mass spectrum. In the α2 mycolic acid structure determined by Gray and colleagues from MSM the methyl branch was assigned to the side of the olefin farther from the ω end of the meromycolate chain based upon similar evidence (*21*). Thus the precursor required for *trans* cyclopropanation would be required to have the methyl branch on the ω side, different than that in the natural α2 series of MSM. This suggested that the methyl transferase that acts upon the *cis* olefinic precursor of the *trans* olefinic substrate for cyclopropanation must add the methyl branch to the opposite side of the double bond in these two species. To evaluate this hypothesis and to investigate the precision of double bond and cyclopropane introduction into mycolic acids in MTB we examined ^{13}C-labeling patterns from specifically labeled biosynthetic precursors.

EXPERIMENTAL PROCEDURES

Materials and strains. Cultures were grown in Middlebrook 7H9 ADC (Difco) supplemented with 2 ml/l glycerol, 0.5 ml/l Tween 80, 5 g/l bovine serum albumin (Fraction V, Sigma), 2 g/l glucose, 14 mM sodium chloride, and contained 25 μg/ml kanamycin where appropriate. *Mycobacterium tuberculosis* H37Rv (ATCC 27294) or H37Rv containing pMV206__mmal (*22*) were cultured in 500 ml roller bottle flasks at 37°C in a Biosafety Level 3 laboratory. *Mycobacterium smegmatis* strain mc^{2}155 (*23*) was grown at 37°C in an Erlenmyer flask with shaking.

Isotopic labeling and purification of mycolic acid methyl esters. Final concentrations for ^{13}C labeling were 3 mg/ml for [1-^{13}C]acetate or [2-^{13}C]acetate, and 0.6 mg/ml for [*methyl*-^{13}C]-L-methionine (Cambridge Isotope Laboratories, Andover, MA). For the preparation of mycolic acid methyl esters, cell pellets were hydrolized in 15% tetrabutylammonium hydroxide overnight at 80°C (*24*). An equal volume of methylene chloride was added and methyl esters were prepared by the addition of 0.008 volumes methyl iodide and incubation at room temperature for 2 h with constant mixing. The organic phase was washed twice with 0.1 M HCl and twice with water and then dried at 70°C under a stream of air. This was then redissolved in 2 ml toluene plus 1 ml acetonitrile and the methyl mycolates precipitated with the addition of 2 ml acetonitrile followed by incubation at −20°C overnight (*25*). Precipitated mycolic acid methyl esters were collected by centrifugation at 8000*g* for 40 min at 4°C, dried, and either subjected to NMR (in the case of *M. smegmatis*) or further purified by preparative thin layer chromatography. For preparative TLC, 3 to 11 mg of precipitated mycolic acid methyl esters were dissolved in 0.5 ml dichloromethane and applied to a 20 × 20 cm 1000-μm silica gel plate (Analtech). The plate was developed five times with hexane:ethyl acetate 95:5 (v:v) using fresh solvent each time. The plate was allowed to air dry approximately 30 min between developments. Alpha, methoxy and keto mycolic acid methyl esters (from highest to lowest mobility, respectively) were visualized by spraying the plate with 0.01% rhodamine 6G in ethanol. The mycolic acid methyl ester bands were scraped off the TLC, eluted three times with diethyl ether, and filtered through a 0.2-μm PVDF filter. These samples

were then dried and subjected to NMR analysis. Typical yields were 0.3–1.3 mg purified mycolic acid methyl ester per 100 ml culture.

NMR experiments. NMR experiments were performed on a Bruker 500 MHz spectrometer in $CDCl_3$. Carbon-13 NMR spectra were acquired in proton uncoupled mode. Unlabeled purified ketomycolic acid methyl ester from H37Rv (11.7 mg) was subjected to HMQC in $CDCl_3$ using the pulse sequence previously described (*26*).

RESULTS

Determination and assignment of the ^{13}C-NMR and HMQC spectrum of ketomycolic acid from MTB. Total mycolic acids from MTB strain H37Rv were prepared by hydrolysis with tetrabutylammonium hydroxide and methyl esterified with methyl iodide. As described previously for H37Rv, the amount of *trans*-mycolate was small (*15*). Thus, these mycolic acid methyl esters were found to be composed of a mixture of approximately 45% alpha, 45% methoxy, and 10% ketomycolic acids (Fig. 1, note that *cis* and *trans* isomers are not resolved by normal phase chromatography). A large amount of keto mycolic acid methyl ester (11.7 mg) was purified from this mixture by preparative thin layer chromatography and subjected to heteronuclear correlation spectroscopy to facilitate assignment of the carbon resonances (Fig. 3). In the proton spectrum *trans*-cyclopropanes exhibit a low-intensity complex multiplet at δ ^{1}H 0.0 to 0.2 ppm, while *cis* cyclopropanes show peaks at δ ^{1}H −0.33, 0.57, and 0.64 ppm.

Using the HMQC spectrum we were able to assign proton and carbon peaks for many of the carbons of the ketomycolate backbone including carbons alpha to the ketone (δ ^{13}C 41.37 and 46.55 ppm), the alpha methyl branch (δ ^{13}C 16.60 ppm), and the *cis* cyclopropane (δ ^{13}C 16.00 and 11.14 ppm) (see Fig. 3). The methyl ester moiety, as well as the alpha alkyl and beta hydroxy nuclei relative to the methyl ester were also readily identified (the chemical shift values are summarized in Table 1).

Position-specific [^{13}C]-acetate incorporation into MTB and MSM mycolic acids. To compare the pattern of incorporation of acetate precursors with that predicted from the standard model of fatty acid biosynthesis we labeled mycolic acids by growth of the appropriate microorganism in the presence of [1-^{13}C] or [2-^{13}C] acetic acid. The mero chain and alpha branch are thought to be biosynthesized by normal lipid biosynthetic enzymes involving repeated steps of malonyl-CoA condensation and decarboxylation, resulting in elongation by two carbons (*4*). Malonyl-CoA is biosynthetically produced by carboxylation of acetyl-CoA at the 2-carbon. During fatty acid elongation the 2-carbon of malonyl-CoA becomes attached to the 1-carbon of the growing acyl chain thus a 1–2 order is preserved in the intact molecule and labeling patterns can be predicted. The predicted labeling patterns for the *trans* ketomycolate of MTB and the α2 mycolate of MSM with [1-^{13}C]-acetate are shown in Fig. 1.

To examine the biosynthesis of the *trans* mycolic acids produced in MTB that are normally produced only in low abundance, we utilized H37Rv (pMV206__*mma1*) a strain of MTB that overexpresses the *mma1* gene (*15*). This gene encodes an MTB mycolic acid methyltransferase enzyme which converts the proximal *cis* olefin to *trans* with an allylic methyl branch. Overexpression of this enzyme results in a dramatic increase in the amount of *trans* mycolic acid and the appearance of the alpha-methyl *trans* olefinic precursor. Since ketomycolates contain the highest proportion of *trans* species in MTB we purified this class of mycolates by preparative TLC and

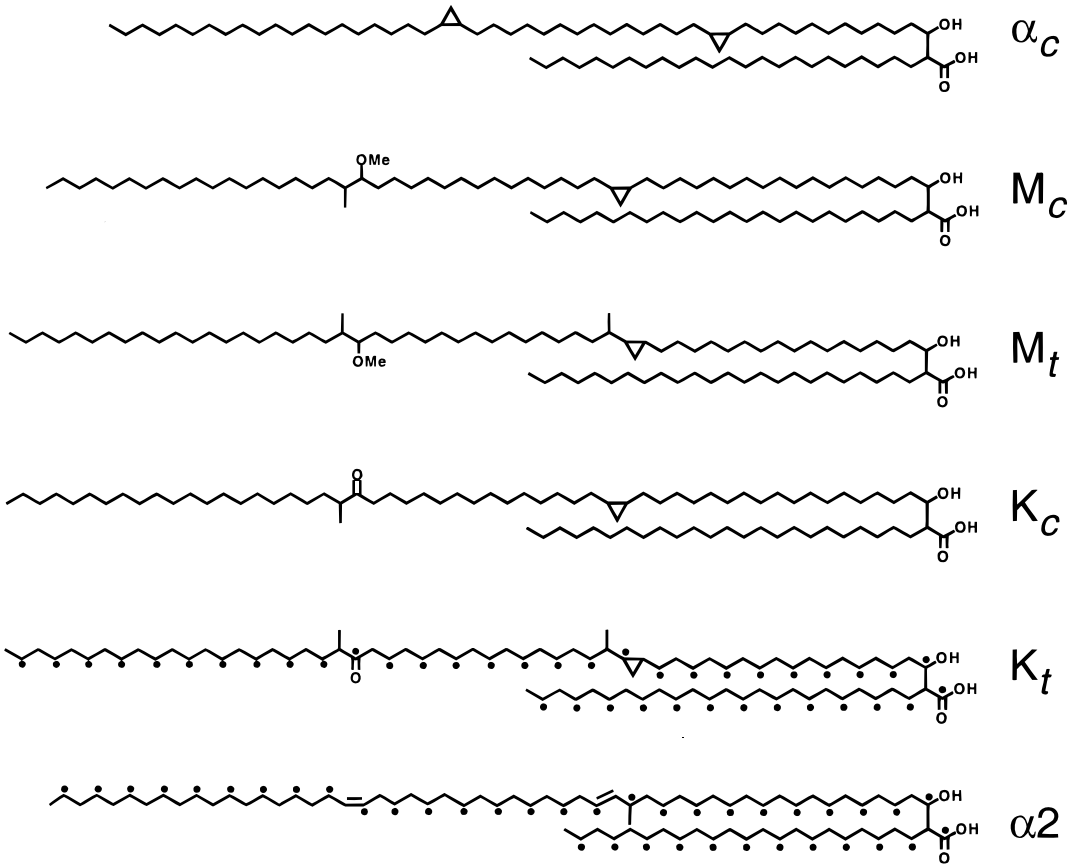

FIG. 1. Structures of alpha, methoxy, and keto mycolic acids from *M. tuberculosis* are labeled α, M, and K, respectively. The subscript *c* or *t* indicates *cis* or *trans* conformation of the promixal cyclopropane. *Trans* alpha mycolate has not been observed. The structure of *M. smegmatis* α2 mycolic acid is also shown for comparison. Note the position of the methyl branch relative to the cyclopropane or double bond. Dots represent which carbons are predicted to be metabolically labeled with [1-^{13}C]acetic acid.

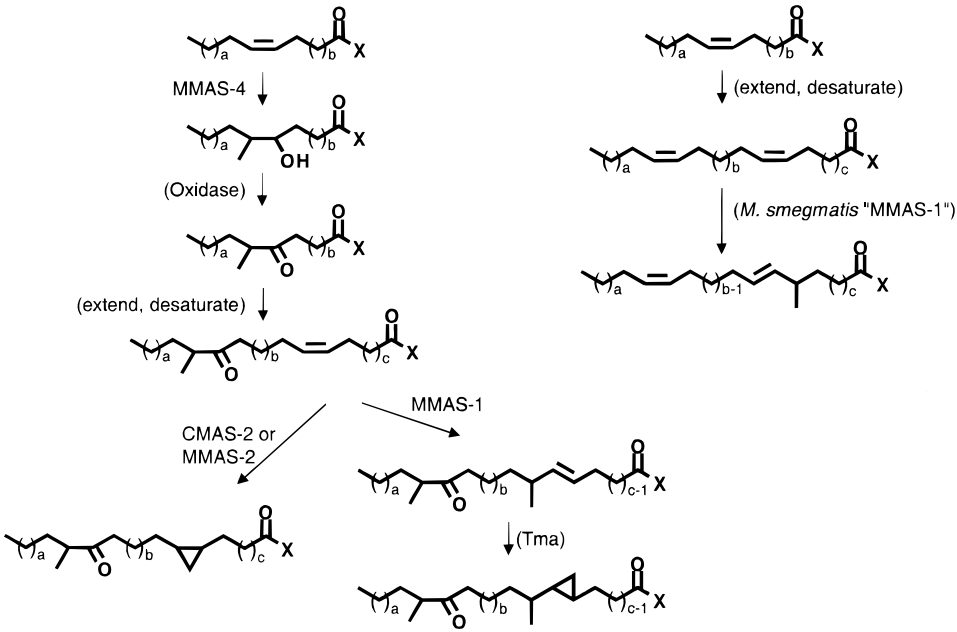

FIG. 2. Partial biosynthetic scheme for selected mycolic acids. Left, pathway for keto mycolic acid synthesis in *M. tuberculosis*. Right, pathway for $\alpha 2$ mycolic acid synthesis in *M. smegmatis*. Steps for enzymes that have not yet been identified are in parentheses. X indicates the lipid carrier during synthesis, most likely this is the acyl carrier protein AcpM (31).

subjected it to ^{13}C NMR. Total mycolates from wild type MSM were also used in a separate ^{13}C NMR experiment since the *trans* olefinic species is naturally the most abundant species under normal growth conditions (*12*).

Comparison of the [1-^{13}C]- and [2-^{13}C]-labeled mycolates from the two species reveals several striking features (summarized in Table 1). The *cis* and *trans* olefinic carbons in the MSM mycolic acids were readily apparent in the downfield portion of the spectrum. The *cis* olefinic carbon signal at δ ^{13}C 130.13 ppm was observed with both 1- and 2-labeled samples as expected since the carbon atoms are magnetically equivalent. However, the chemical shifts for the assymetric *trans* olefinic carbons present in the $\alpha 2$ mycolates reveal a strict positional specificity. The methyl branch allylic to the *trans* double bond creates an 8 ppm difference in the signals for the two olefinic nuclei, with resonances at δ ^{13}C 136.69 and δ ^{13}C 128.65 for carbons alpha and beta to the methyl branch, respectively. Strikingly, the 136.69 signal is seen only in the [2-^{13}C] acetate-labeled sample of mycolates from MSM, while the 128.65 peak is present only in the [1-^{13}C]-labeled mycolates. This demonstrates that the placement of the *trans* double bond occurs between carbons $\omega - n$ and $\omega - (n + 1)$, where n is an odd integer, never when n is an even integer. The *trans* olefinic species observed in *M. tuberculosis* (pMV206__*mma1*) showed the opposite pattern of labeling with [1-^{13}C] acetate labeling the carbon at δ ^{13}C 136.69 and [2-^{13}C] acetate labeling the carbon at δ ^{13}C 128.65. Thus the methyltransferase responsible for addition

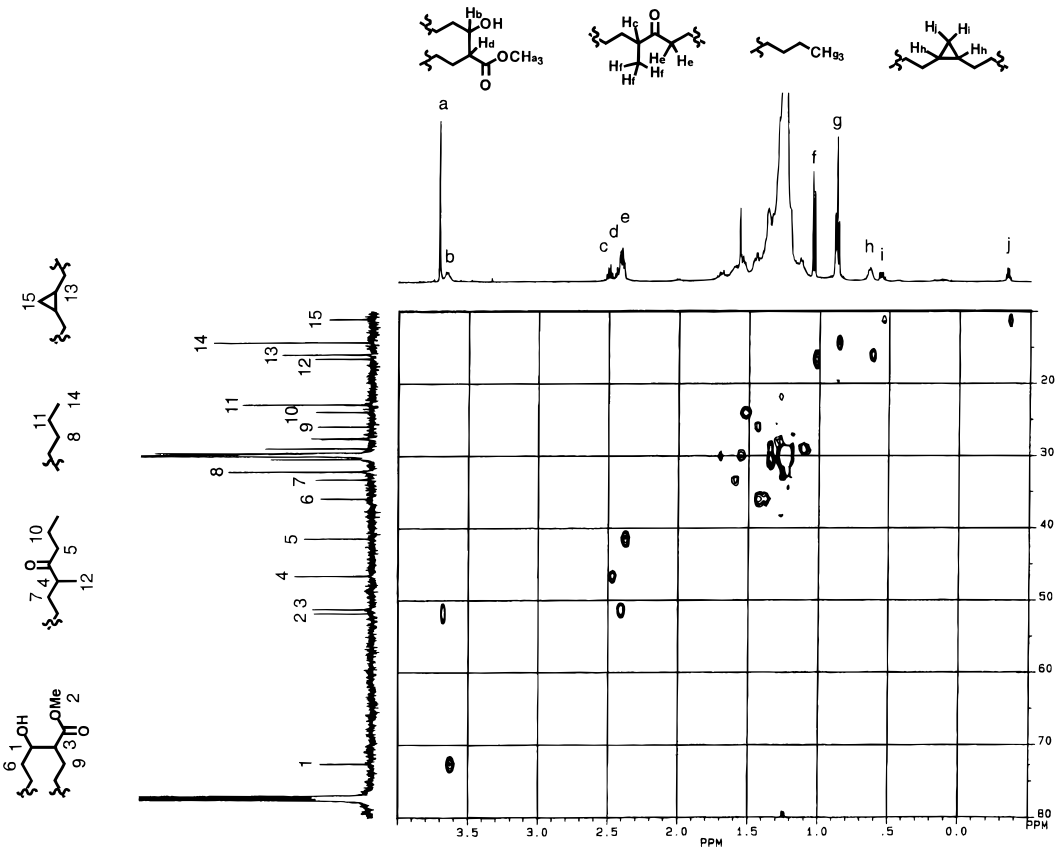

FIG. 3. Heteronuclear correlation spectrum (HMQC) of *M. tuberculosis* ketomycolic acid methyl ester in deuterochloroform. A detail of the most informative portion of the spectrum is shown, with selected carbon peaks numbered and identified on the substructures to the left and selected proton peaks lettered and identified on the substructures above.